

PATENT COOPERATION TREATY

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Assistant Commissioner for Patents
 United States Patent and Trademark
 Office
 Box PCT
 Washington, D.C.20231
 ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 11 May 2000 (11.05.00)	Applicant's or agent's file reference 1770-214PCT
International application No. PCT/CA99/00895	Priority date (day/month/year) 28 September 1998 (28.09.98)
International filing date (day/month/year) 27 September 1999 (27.09.99)	
Applicant KARAPLIS, Andrew, C. et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:

10 April 2000 (10.04.00)

☐ in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was
☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

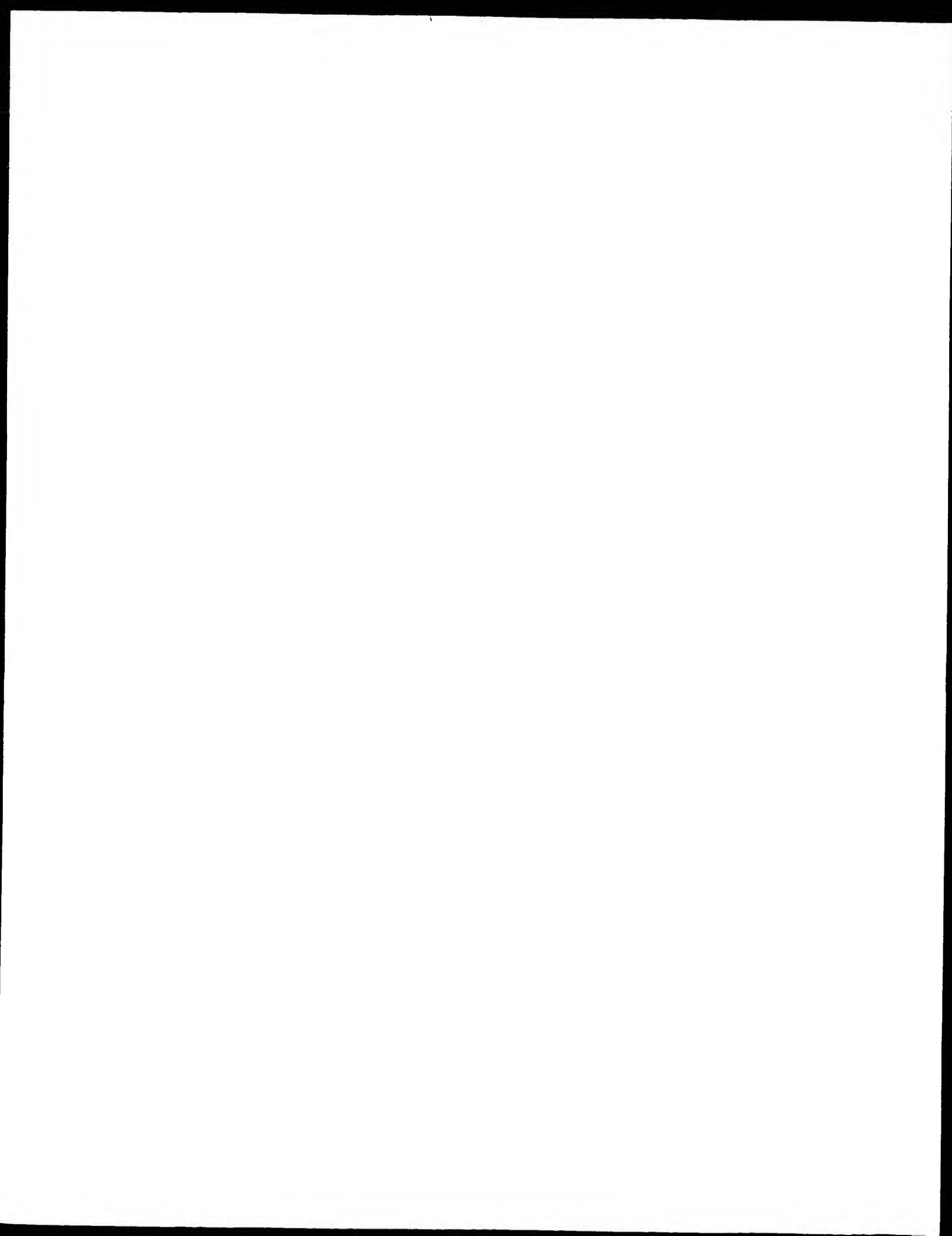
The International Bureau of WIPO
 34, chemin des Colombettes
 1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Claudio Borton

Telephone No.: (41-22) 338.83.38



PATENT COOPERATION TREATY

PCT

NOTICE INFORMING THE APPLICANT OF THE
COMMUNICATION OF THE INTERNATIONAL
APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

From the INTERNATIONAL BUREAU

To:

COTE, France
Swabey Ogilvy Renault
Suite 1600
1981 McGill College Avenue
Montréal, Québec H3A 2Y3
CANADASWABEY OGILVY RENAULT
MCGILL COLLEGE
RECEIVED

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A.M.

7 8 9 10 11 12 1 2 3 4 5 P.M.

Date of mailing (day/month/year) 06 April 2000 (06.04.00)		
Applicant's or agent's file reference 1770-214PCT		IMPORTANT NOTICE
International application No. PCT/CA99/00895	International filing date (day/month/year) 27 September 1999 (27.09.99)	
		Priority date (day/month/year) 28 September 1998 (28.09.98)
Applicant MCGILL UNIVERSITY et al		

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
AU,CN,JP,KP,KR,US

In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
AE,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CR,CU,CZ,DE,DK,DM,EA,EE,EP,ES,FI,GB,GD,GE,
GH,GM,HR,HU,ID,IL,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MD,MG,MK,MN,MW,MX,NO,NZ,OA,
PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,ZA,ZW
The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
06 April 2000 (06.04.00) under No. WO 00/18954

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, a demand for international preliminary examination must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the national phase, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer J. Zahra
Facsimile No. (41-22) 740.14.35	Telephone No. (41-22) 338.83.38



DEC 8 2000

PCT 8 9 10 11 12 1 2 3 4 5

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

Côté, France
SWABEY OGILVY RENAULT
1981 McGill College Avenue
Suite 1600
Montréal, Québec H3A 2Y3
CANADA

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL PRELIMINARY
EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing
(day/month/year) 01.12.2000

Applicant's or agent's file reference
1770-214PCT

IMPORTANT NOTIFICATION

International application No.
PCT/CA99/00895

International filing date (day/month/year)
27/09/1999

Priority date (day/month/year)
28/09/1998

Applicant
MCGILL UNIVERSITY et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/



European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Authorized officer

Saavedra Martinez, V

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PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 1770-214PCT	<div style="display: flex; justify-content: space-between;"> <div>FOR FURTHER ACTION</div> <div>See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)</div> </div>	
International application No. PCT/CA99/00895	International filing date (day/month/year) 27/09/1999	Priority date (day/month/year) 28/09/1998
International Patent Classification (IPC) or national classification and IPC C12Q1/68		
Applicant MCGILL UNIVERSITY et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.


2. This REPORT consists of a total of 10 sheets, including this cover sheet.

- ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand 10/04/2000	Date of completion of this report 01.12.2000
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Giry, M Telephone No. +49 89 2399 7328



INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/CA99/00895

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-9,11-30	as originally filed	
10	with telefax of	07/09/2000

Claims, No.:

1-13	with telefax of	07/09/2000
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Drawings, sheets:

1/18-18/18	as originally filed
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2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/CA99/00895

- ☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:
see separate sheet

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

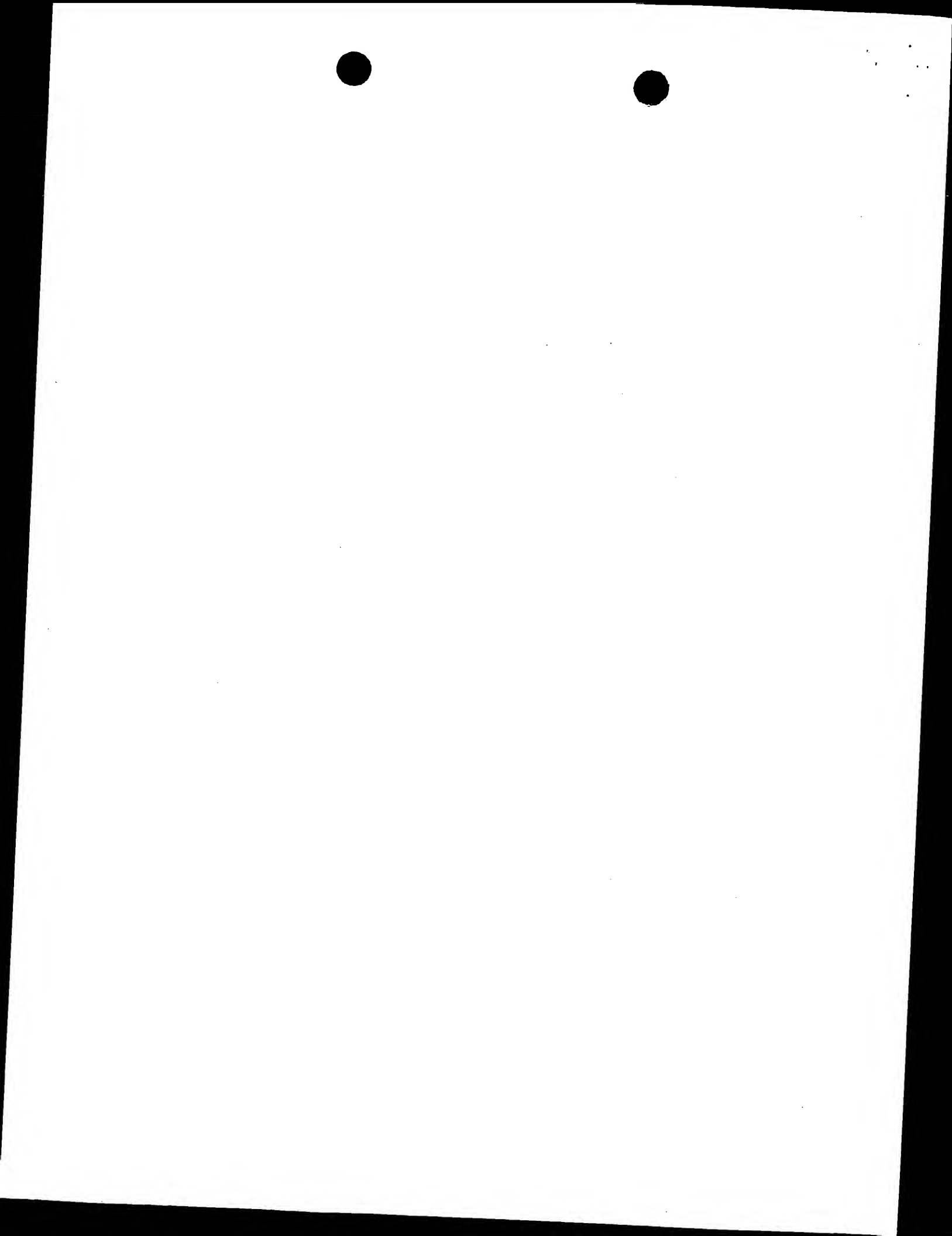
The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

- ☐ the entire international application.
☒ claims Nos. 3-4, 7-10.

because:

- ☒ the said international application, or the said claims Nos. 3-4, 7-10 relate to the following subject matter which does not require an international preliminary examination (*specify*):
see separate sheet
- ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):
- ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.
- ☐ no international search report has been established for the said claims Nos. .
2. A meaningful international preliminary examination report cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:
- ☐ the written form has not been furnished or does not comply with the standard.
☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement



**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/CA99/00895

1. Statement

Novelty (N)	Yes: Claims 1-13
	No: Claims
Inventive step (IS)	Yes: Claims
	No: Claims 1-13
Industrial applicability (IA)	Yes: Claims 1-2, 11-13
	No: Claims

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/CA99/00895

Re Item I

Basis of the report

1. Sequence listing pages 1-11 filed together with the original application documents are also included in the basis of the opinion.
2. The applicant has requested deletion of Figures 6B and 6C ; however, it is not possible for the IPEA to introduce amendments in the application documents.

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

Claims 3-4 and 7-10 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Art. 34(4)(a)(i) PCT). See Item V-4 below.

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1 - **Reference** is made to the following documents :

D1 : WO 98 10078 A, 12 March 1998

D2 : Lipman ML et al. : 'Cloning of human PEX cDNA : Expression, subcellular localization, and endopeptidase activity.' J. Biol. Chem. (1998), vol. 273, no. 22, pages 13729-13737

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/CA99/00895

2 - Novelty - Art. 33(1) and (2) PCT :

- 2.1 The subject-matter of claims 1-2 appears to be new since none of the prior art documents disclose a method for the diagnosis of metabolic bone diseases based on determining the level of PTHrP in a biological sample.
- 2.2 A compound that modulates PTH and PTHrP levels for the modulation of PEX enzymatic activity in a method for the treatment of metabolic bone diseases and its utilization for the manufacture of a medicament allowing said treatment have not been mentioned in the available prior art documents. Therefore, claims 3-6 can be considered as novel.
- 2.3 The modulation of PTH and PTHrP levels that regulate osteoblasts activity in a method for the treatment of metabolic bone diseases and the use of a compound able to modulate said levels for achieving said treatment is not described in the prior art documents. Thus, claims 7-10 can be regarded as new.
- 2.4 None of the prior art documents disclose a non-human transgenic mammal bearing the PEX gene sequence under the control of a proximal promoter of a pro- α 1(I) collagen gene. Therefore, the subject-matter of claims 11-13 appears to be new.

3 - Inventive step - Art. 33(1) and (3) PCT :

- 3.1 The closest prior art document D1 concerns the cloning of the human PEX cDNA. Document D1 mentions that the PEX gene encodes an endopeptidase involved either in activation or degradation of a number of peptide hormones and also discloses the use of the PEX active site in the treatment and diagnosis of hypophosphatemic disorders (p. 2, lines 34-35 and p. 3, lines 4-5, respectively). A method for the design of drugs to be used as competitive inhibitors or activators (*i.e.* modulators) of PEX enzymatic activity in cases of hypophosphatemia is described (p. 3, lines 23-27). Finally, the said document mentions a transgenic mouse bearing the PEX gene (p. 4, lines 26-28).

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/CA99/00895

- 3.2 The subject-matter of claims 1-2 and 7-10, which concerns methods for diagnosis and treatment of metabolic bone diseases, differs from D1 in that said methods are based on the quantification of the PTHrP level (the former) or PTH and PTHrP levels (the latter). The problem to be solved by the present invention may therefore be regarded as providing methods for the diagnosis and treatment of metabolic bone diseases. However, document D2 demonstrates that PEX enzymatic activity is involved in the degradation of PTH, thereby modulating the level of this peptide affecting osteoblasts activity (p. 13737, col. 1, lines 50-61). Therefore, the skilled person would regard it a normal design procedure to include the determination of PTH or PTHrP levels in the method described in D1 in order to diagnose metabolic bone diseases, thereby arriving at the method disclosed in claims 1-2. Moreover, the administration of a drug in order to modulate the level of a compound whose deregulation is responsible for a disease comes within the scope of the customary practice followed by persons skilled in the art, especially as the advantages thus achieved can readily be foreseen. Consequently, the subject-matter of claims 1-2 and 7-10 does not involve an inventive step.
- 3.3 The applicants representative argues that the present invention cannot be considered as obvious to one skilled in the art since it was not obvious to the present applicants who are experts in the field. This argument cannot be accepted since the fact that PTHrP, which is important in the development of normal bone density, shares many of the structural features of PTH and may therefore serve as a substrate for PEX (see the description, p. 8, lines 24-28), is implicitly known from the prior art. Thus, studying PTHrP in the context of bone diseases and in view of the obtained results, incorporate a compound that modulate its level in a method for the treatment of bone disease is an obvious design procedure that does not require the exercise of inventive skill.
- 3.4 Claims 3-6 disclose the use of a compound for the modulation of PEX enzymatic activity in a method for the treatment of metabolic bone diseases and for the manufacture of a medicament. As the methods provided in document D1 concern hypophosphatemia disorders in general (p. 3, lines 23-27), claims 3-6 only differ from D1 in that the mentioned methods are only used for the treatment of metabolic bone diseases. However, this selection cannot be regarded as inventive because one of the hypophosphatemia disorders mentioned in D1 is OHO. Thus,

the skilled person is given a hint in D1 that the method disclosed therein may be used for the treatment of osteomalacia. Therefore, no inventive step can be acknowledged for claims 3-6.

- 3.5 Claims 11-13 concerning a non-human transgenic animal differ from D1 by the insertion of the PEX gene under the control of a proximal promoter of a pro- $\alpha 1$ (I) collagen gene (claim 11), the said promoter being of a murine origine (claim 12) and constituted by a 2.3 kb fragment thereof (claim 13). These technical features have been mentioned neither in the description, nor in the available prior art documents. It is therefore not apparent which technical problem is solved by inserting the said promoter. Consequently, claims 11-13 lack inventive step. The applicants' representative argues that the teachings of claims 11-13 appear on p. 5 lines 1-14 of the description. This argument cannot be accepted since this paragraph consists merely in a paraphrase of the claims without any further information, and thus cannot be considered as adequate support.

4 - Industrial applicability - Art. 33(1) and (4) PCT :

For the assessment of the present claims 3-10 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment. See also PCT Guidelines IV-2.5.

The subject-matter of claims 1-2 and 11-13 appears to be industrially applicable.

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/CA99/00895

5 - P documents

The document "Schneider HG et al : 'Parathyroid hormone-related protein mRNA and protein expressions in multiple myeloma: A case report.' J. Bone Mineral Research, 1998, vol. 13, no. 10, pages 1640-1643", was published after the priority date, but before the filing date of the present application and is therefore relevant only for those parts, if any, of the present application which do not have a valid claim to priority.

Re Item VIII

Certain observations on the international application

- a - The features of claims 11-13, that the PEX gene sequence is placed under the control of a proximal promoter of a pro- $\alpha 1$ (I) collagen gene (claim 11), that the said promoter is of a murine origine (claim 12) and that the collagen gene is a 2.3 kb fragment thereof (claim 13) lack support in the description (see point 3.5) (Art. 6 PCT).
- b - The application does not contain any technical data which prove that patient suffering from metabolic bone diseases present altered PTHrP levels. Hence, claims 1 and 2 are not supported by the description (Art. 6 PCT):
- c - The term "metabolic bone diseases" is vague and it is not clear whether it encompasses diseases leading to osteolysis, such as bone metastases. The definition given in the description on p. 5, lines 17-19, due to the expression "without limitation" (p. 5, line 18) does not provide any explanation (Art. 6 PCT).
- d - The statement "levels that regulate osteoblast activity" in claims 7 and 9 lacks support in the description. The description does not describe how the levels that regulate osteoblast activity can be regulated separately. It is therefore not clear whether the level that regulates osteoblast activity also regulates osteoclast activity. The passage on p. 8, lines 24-35, pointed out by the applicants

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/CA99/00895

representative does not provide any explanation about this issue (Art. 6 PCT).

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12Q 1/68	A2	(11) International Publication Number: WO 00/18954 (43) International Publication Date: 6 April 2000 (06.04.00)
(21) International Application Number: PCT/CA99/00895 (22) International Filing Date: 27 September 1999 (27.09.99) (30) Priority Data: 2,245,903 28 September 1998 (28.09.98) CA (71) Applicant (for all designated States except US): MCGILL UNIVERSITY [CA/CA]; Office of Technology Transfer, 845 Sherbrooke Street West, Montréal, Québec H3A 2T5 (CA). (72) Inventors; and (75) Inventors/Applicants (for US only): KARAPLIS, Andrew, C. [CA/CA]; 95 Meaney, Kirkland, Québec H9J 3V6 (CA). GOLTZMAN, David [CA/CA]; 667 Belmont, Westmount, Québec H3Y 2W3 (CA). LIPMAN, Mark, L. [CA/CA]; 2258 Fulton Road, Town of Mount Royal, Québec H3R 2L4 (CA). HENDERSON, Janet, E. [CA/CA]; 70 Woseley, Montreal West, Québec H4X 1V7 (CA). (74) Agents: COTE, France et al.; Swabey Ogilvy Renault, Suite 1600, 1981 McGill College Avenue, Montréal, Québec H3A 2Y3 (CA).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: USE OF PEX IN THE TREATMENT OF METABOLIC BONE DISEASES (57) Abstract <p>The present invention relates to a method for the diagnosis of metabolic bone diseases in a patient, which comprises the step of determining the level of PTHrP in a biological sample of a patient wherein an alteration of PTHrP levels from that of a normal individual is indicative of metabolic bone diseases and/or metabolic bone diseases predisposition. The present invention also relates to a method for the diagnosis of metabolic bone diseases in a patient, which comprises the step of determining the level of PTHrP in a biological sample of a patient wherein an alteration of PTHrP levels from that of a normal individual is indicative of metabolic bone diseases and/or metabolic bone diseases predisposition.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
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JC08 Rec'd PCT/PTO 28 MAR 2001

USE OF PEX IN THE TREATMENT OF METABOLIC BONE DISEASESBACKGROUND OF THE INVENTION(a) Field of the Invention

5 The invention relates to the use of *PEX* in the treatment of metabolic bone diseases, such as osteomalacia and osteoporosis.

(b) Description of Prior Art

10 Mutations in the *PEX* gene are responsible for X-linked hypophosphatemic rickets (HYP). To gain insight into the role of *PEX* in normal physiology we have cloned the human full-length cDNA and studied its tissue expression, subcellular localization, and peptidase activity. We show that the cDNA encodes a 749 amino
15 acid protein structurally related to a family of neutral endopeptidases that include neprilysin (NEP) as prototype. By Northern blot analysis, the size of the full-length *PEX* transcript is 6.5 kb. *PEX* expression, as determined by semi-quantitative PCR, is high in bone
20 and in tumor tissue associated with the paraneoplastic syndrome of renal phosphate wasting. *PEX* is glycosylated in the presence of canine microsomal membranes and partitions exclusively in the detergent phase from Triton X-114 extractions of transiently transfected COS
25 cells. Immunofluorescence studies in A293 cells expressing *PEX* tagged with a c-myc epitope show a predominant cell-surface location for the protein with its C-terminal domain in the extracellular compartment, substantiating the assumption that *PEX*, like other members of the neutral endopeptidase family, is a type II
30 integral membrane glycoprotein. Cell membranes from cultured COS cells transiently expressing *PEX* efficiently degrade exogenously added PTH-derived peptides, demonstrating for the first time that recombinant *PEX*
35 can function as an endopeptidase. *PEX* peptidase activ-

ity may provide a convenient target for pharmacological intervention in states of altered phosphate homeostasis and in metabolic bone diseases.

5 X-linked hypophosphatemic rickets (HYP) is the most common inherited disorder of renal phosphate wasting characterized by severe hypophosphatemia, renal phosphate wasting, reduced serum concentrations of 1,25-dihydroxyvitamin D levels, and defective bone mineralization. Until recently, much of our understanding
10 of HYP has been facilitated by the availability of two murine homologues, the *Hyp* and *Gy* mice, which exhibit many of the phenotypic features of HYP. Through positional cloning, however, a gene which spans the deleted region Xp22.1 in HYP patients, or is mutated in
15 non-deletion patients with the disorder, was identified (designated *PEX*) and its partial cDNA sequence reported (The HYP Consortium (1995) *Nature Genetics* **11**, 130-136). The predicted human *PEX* gene product, as well as its murine homologue (Du, L. et al. (1996) *Genomics* **36**,
20 22-28), exhibit homology to a family of neutral endopeptidases involved in either activation or degradation of a number of peptide hormones. It has been postulated that *PEX* metabolizes a peptide hormone that modulates renal tubular phosphate handling. Such an activity
25 could involve either the processing of a phosphate-reabsorbing hormone precursor to its active form or the inactivation of a circulating phosphaturic factor. These speculations notwithstanding, the physiologic function of the *PEX* gene product and the mechanisms
30 that lead to the renal and skeletal abnormalities of HYP remain to be defined.

Oncogenous hypophosphatemic osteomalacia (OHO) is a rare acquired disorder of phosphate homeostasis with biochemical and physical abnormalities similar to
35 HYP. This syndrome is associated with a variety of his-

tologically distinct, usually benign, mesenchymal tumors whose excision promptly reverses the metabolic abnormalities and results in cure of the bone disease. It is generally thought that a factor(s) produced by these tumors promotes phosphaturia and inhibits the renal conversion of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D. The nature of the phosphaturic substance remains unknown and is likely distinct from both parathyroid hormone (PTH) and calcitonin, two polypeptide hormones known to inhibit the renal tubular reabsorption of phosphorus. Because of the striking similarity in the clinical presentation of patients with OHO and HYP, it is postulated that the factor causing phosphaturia in OHO is the active form of the PEX substrate. The identification and characterization of the putative PEX substrate, referred to as phosphatonin, however, will require first a better understanding of PEX function.

To date, there is still a need to understand how local factors produced in the bone regulate bone formation and bone resorption. Derangement of these factors leads to metabolic bone diseases. Pharmacological manipulation of such factors may serve as a novel approach to the treatment of these disorders.

It would be highly desirable to be provided with a tool in the treatment of metabolic bone diseases, such as osteomalacia and osteoporosis.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide a tool in the treatment of metabolic bone diseases, such as osteomalacia and osteoporosis.

Another aim of the present invention is to provide the use of PEX in the treatment of metabolic bone diseases, such as osteomalacia and osteoporosis.

Another aim of the present invention is to provide a method of diagnostic of metabolic bone diseases, such as osteomalacia and osteoporosis.

5 Toward this objective, we have cloned a cDNA encoding the full-length human *PEX* protein, and determined the tissue distribution of *PEX* transcripts. In addition, we have examined the subcellular localization of recombinant *PEX* protein and demonstrated its peptidase activity.

10 In accordance with the present invention there is provided a method for the diagnosis of metabolic bone diseases in a patient, which comprises the step of determining the level of PTHrP in a biological sample of a patient wherein an alteration of PTHrP levels from
15 that of a normal individual is indicative of metabolic bone diseases and/or metabolic bone diseases predisposition.

20 In accordance with the present invention there is provided a method for the treatment of metabolic bone diseases, which comprises administering to a patient a compound for the modulation of *PEX* enzymatic activity.

25 In accordance with the present invention there is provided the use of a compound for the modulation of *PEX* enzymatic activity for the manufacture of a medication for treating metabolic bone diseases.

30 In accordance with the present invention there is provided a method for the treatment of metabolic bone diseases, which comprises modulating PTH and PTHrP levels that regulate osteoblast activity in a patient to modulate bone breakdown and bone formation.

35 In accordance with the present invention there is provided the use of modulation of PTH and PTHrP levels that regulate osteoblast activity for the treatment of metabolic bone diseases.

In accordance with the present invention there is provided a non-human transgenic mammal to study the role of PEX in bone development and homeostasis, whose germ cells and somatic cells contain a PEX gene construct for expression of PEX in osteoblast consisting essentially of a recombinant PEX gene sequence under the control of a proximal promoter of a pro- α 1(I) collagen gene, the PEX gene construct being introduced into the mammal, or an ancestor of the mammal, at an embryonic stage.

The non-human mammal is preferably a mouse and the proximal promoter is preferably murine pro- α 1(I) collagen gene, more preferably a 2.3 kb fragment thereof.

For the purpose of the present invention the following terms are defined below.

The expression "metabolic bone diseases" includes, without limitation, osteomalacia, osteoporosis, osteopetrosis and Paget's disease.

20

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates PEX mRNA expression in OHO tumors;

Fig. 2A illustrates human PEX cDNA cloned from OHO tumors (SEQ ID NOS:1-2);

Fig. 2B illustrates human PEX and human NEP protein alignment (SEQ ID NOS:3-4);

Fig. 2C illustrates the Tmpred output for PEX;

Fig. 3 illustrates PEX expression in human tissues;

30

Fig. 4 illustrates a Northern blot analysis of PEX mRNA;

Fig. 5 illustrates *in vitro* translation of human PEX cRNA;

Figs. 6A-6B illustrate TRITON™ X-114 extraction and immunofluorescent localization of PEX;

Figs. 7A-7C illustrate HPLC analysis of the hydrolysis of [D-Ala²,Leu⁵]enkephalin;

5 Figs. 8A-8C illustrate the hydrolysis of PTH-derived peptides by PEX endopeptidase activity; and

Fig. 9 illustrates Schematic representation of phosphate handling in the proximal renal tubule in normal, OHO, and HYP states.

10

DETAILED DESCRIPTION OF THE INVENTION

PEX is a Cell Membrane-Associated Protein

Previous studies have established that NEP, ECE-1
15 and Kell blood group glycoprotein are integral membrane proteins. We have used extraction with the detergent TRITON™ X-114 and immunochemical localization to examine whether PEX is also a membrane-associated protein. For
20 identification of PEX, we generated a construct in which the carboxyl terminus sequences of PEX are modified by a human c-myc tag. The epitope tag was inserted immediately upstream of the potential prenylation motif so that any lipid modification of the PEX protein may proceed uninter-

25 TRITON™ X-114 is a detergent that forms an aqueous solution at 4°C but separates into hydrophobic and aqueous phases when the temperature is raised to 30-37°C. This property has been used as an indicator of the hydrophobic nature of proteins, with integral membrane proteins
30 partitioning exclusively in the detergent phase while highly hydrophilic proteins associate with the aqueous phase. TRITON™ X-114 extracts from COS-7 cells transiently expressing PEX tagged with the c-myc epitope showed that PEX partitions nearly exclusively into the
35 detergent phase. This finding indicates that PEX is a

membrane-associated protein and is consistent with the prediction from sequence analysis that it is an integral membrane protein.

To determine the subcellular localization of PEX, the distribution of recombinant protein expressed in stably transfected A293 cells was examined using immunofluorescence. When cells were fixed and permeabilized, myc-tagged PEX immunostaining was detected primarily on the cell surface, but in a number of cells staining was also observed intracellularly, although no signal was observed in the nucleus. If permeabilization was omitted, staining was localized exclusively to the plasma membrane, while untransfected cells or cells transfected with vector alone showed no immunofluorescent staining. Since the myc-tag was inserted in the carboxyl end of PEX, these findings further corroborate the sequence-based prediction that PEX is a Type II integral membrane protein with its large C-terminal hydrophilic domain containing the active enzymatic site in the extracellular compartment.

20 **Recombinant PEX protein has peptidase activity**

The subcellular localization and sequence similarity between PEX and NEP strongly suggest that PEX functions as a membrane-bound metallopeptidase. However, no peptidase activity has yet been ascribed to PEX. As shown, when [D-Ala², Leu⁵] enkephalin, used to assay for NEP activity, was incubated with cell membrane preparations from vector-transfected COS cells or COS cells expressing equivalent amounts of recombinant human NEP or PEX proteins, as determined by Western blot analysis, production of Tyr-D-Ala-Gly from the substrate was evident only in NEP-expressing membrane preparations. While the PEX sequence preserves two of the residues critical for catalytic activity of NEP (equivalent to E⁶⁴⁶ and H⁷¹¹), it lacks a residue equivalent to R¹⁰² shown to be crucial for the dipeptidylcarboxypeptidase activity of NEP. Therefore,

unlike NEP, *PEX* has no dipeptidylcarboxypeptidase activity.

To test for peptidase activity of recombinant *PEX*, cell membrane preparations from vector-transfected COS cells or COS cells expressing recombinant *PEX* protein were incubated with human parathyroid hormone PTH (1-34) and PTH (1-38). As shown, *PEX* activity was able to degrade both peptides in a very characteristic pattern. Therefore, *PEX* functions as an endopeptidase, and more specifically we have shown for the first time that it degrades PTH. PTH is the first and only known substrate of *PEX*. These observations make two important points:

PEX is a membrane bound protein with its active enzymatic site in the extracellular compartment. The cells with the highest level of *PEX* expression are the osteoblasts (bone forming cells). These cells are also the site of action of circulating PTH at the level of the bone. PTH stimulates these cells to produce factors (nature unknown) which in turn stimulate other bone cells, specifically the osteoclasts, to break down bone. Since *PEX* likely inactivates PTH in contact with osteoblasts, it would result in decreased stimulation of osteoclasts and therefore less bone breakdown.

Alternatively, osteoblasts produce parathyroid hormone-related peptide, PTHrP, which is important in the development of normal bone density. PTHrP shares many of the structural features of PTH and may therefore also serve as substrate for *PEX*. Our previous studies using PTHrP heterozygous-null mice generated by gene targeting have shown that decreased levels of PTHrP in the skeletal microenvironment lead to a premature form of osteoporosis. *PEX* in osteoblasts may therefore modulate local PTHrP levels and thus bone formation. Inhibition of *PEX* enzymatic activity may allow higher local concentrations of PTHrP and therefore better bone formation.

By examining PTH breakdown fragments, we can now design peptide and non-peptide activators and inhibitors of PEX enzymatic activity.

By modulating PTH and PTHrP levels that regulate osteoblast activity, PEX may play a critical role in the pathogenesis of osteomalacia and osteoporosis. By pharmacological modulation of PEX activity, it will be possible to modulate bone breakdown and bone formation. This would be a totally novel approach to the treatment of these metabolic bone diseases.

Experimental procedure

Tumor Tissues

Patient I was a 55 year-old woman who presented with a two-year history of progressively increasing bone pain and difficulty in walking. X-rays of the lumbosacral spine showed diffuse osteopenia. Biochemical investigation showed the serum calcium level to be normal while serum phosphorus was low (0.41 to 0.57 mmol/L; normal, 0.8-1.6 mmol/L). Alkaline phosphatase was 232 U/L (normal, 30-105 U/L) and tubular reabsorption of phosphate while the patient was hypophosphatemic was decreased to 63% (normal, >80%). A search for a tumor was negative and the patient was treated with 1,25-dihydroxyvitaminD3 and oral phosphate. Five years later a right hand mass was discovered and was surgically removed. On histopathological examination, it was a fibrous hemangioma. Postoperatively, the patient noted increasing strength in her lower extremities and marked decrease in her pain. The serum phosphorus normalized (0.96 mmol/L) and the tubular reabsorption of phosphate improved but did not completely normalize (71-76%). No recurrence of the tumor has been found ten years later.

Patient II was a 21 year old man with classic features of OHO. Resection of a benign extraskeletal

chondroma from the plantar surface of the foot resulted in complete reversal of the biochemical and clinical abnormalities associated with the syndrome.

5 Tumor tissue obtained from these two patients at surgery was frozen immediately in liquid nitrogen and stored at -70°C.

PEX Expression in OHO-Associated Tumors

RNA was extracted from tumor tissue using the RNeasy Total RNA kit (Qiagen, Chatsworth, CA) and reverse transcribed using oligo(dT) primer and Super-
10 script II (BRL) reverse transcriptase for 1 hour at 42°C in a final reaction volume of 30 µl. The resulting cDNA was then amplified using human PEX-specific oligo-nucleotide primers PEX-1 (5'-GGAGGAATTGGTTGAGGGCG -3' SEQ ID NO:5) and PEX-2 (5'-GTAGACCACCAAGGATCCAG -3' SEQ
15 ID NO:6), designed from the published cDNA sequence (1298 and 1807 are the nucleotide positions of the 5' end of the sense and antisense primers, respectively) (The HYP Consortium (1995) *Nature Genetics* **11**, 130-
20 136). Following amplification (35 cycles), an aliquot of the PCR reaction was fractionated on a 1% agarose gel and visualized following staining with ethidium bromide.

Cloning of Full-Length PEX cDNA

25 Cloning of the 5' end of PEX cDNA was accomplished by anchored PCR. Total cellular RNA was extracted from tumor II and mRNA was prepared. 1.5 µg of mRNA was reverse transcribed into cDNA using 100 ng of a PEX-specific antisense oligomer (PEX-2) and 200
30 units of Superscript II (BRL) reverse transcriptase for 1 hour at 42°C in a final reaction volume of 30 µl. The resulting cDNA was size fractionated on a 1% agarose gel and fragments corresponding to >600 bp were purified and resuspended in H₂O. The 3' end of the first
35 strand cDNA was homopolymer tailed with dGTP using 1 µl

of Terminal deoxynucleotidyl transferase (TdT) at 37°C for 30 minutes in a volume of 50 µl. Following heat inactivation of the enzyme, the RNA template was removed by incubation with RNase H and the tailed cDNA was purified by phenol-chloroform extraction followed by ammonium acetate precipitation. The purified tailed cDNA was resuspended in H₂O and an aliquot was used for anchored PCR analysis along with 200 ng of an internal PEX specific antisense primer (PEX-3, 5'-CGTGCCCAAGAACTAGGGTGCCACC-3' (SEQ ID NO:7); nucleotide 98 of the published human cDNA sequence is the 5' end of the primer) and 200 ng of oligodC as the sense primer. Forty cycles of PCR were performed using 0.5 µl of Taq polymerase (Promega Biotec, Madison, WI) in a reaction volume of 50 µl. Cycling parameters were: 1 minute of denaturation at 94°C, 2 minutes of annealing at 55°C and 2 minutes of extension at 72°C. The PCR products were fractionated on a 1% agarose gel and a band of 700 bp was isolated, purified, and ligated into pPCRIII vector (Invitrogen). Following transformation into INVαF' bacteria, clones containing the appropriate size insert were sequenced.

To clone the 3' end of PEX cDNA, an aliquot of an amplified unidirectional cDNA library in pCDNA3 vector (Invitrogen) generated from mRNA obtained from tumor I was grown overnight in LB medium and plasmid DNA extracted. DNA (0.5 µg) was subjected to PCR using a PEX-specific sense oligomer (PEX-1) and an antisense oligomer corresponding to the SP6 RNA polymerase binding site sequences present in the pCDNA3 vector. Thirty-five cycles of amplification were performed in a 50 µl reaction volume with each cycle consisting of 1 min denaturation at 94°C, 1 min annealing at 55°C and 1 min extension at 72°C. Amplified products were fractionated on a 1% agarose gel and a 1.2 kb fragment cor-

responding to the 3' end of *PEX* cDNA was subcloned and sequenced.

For expression studies, an *EcoRV* (in the polylinker of pPCRII) /*AccI* (in the *PEX* sequence) fragment containing the 5' end of *PEX* cDNA was ligated into the pPCRII vector containing the 3' end of *PEX* cDNA following digestion with *AccI* and *EcoRV*. The resulting plasmid was restricted with *KpnI* and *NotI* excising the full length *PEX* cDNA that was then inserted into pCDNA3 vector digested at the *KpnI/NotI* sites in the polylinker region, resulting in plasmid p*PEX*. The full-length *PEX* cDNA was sequenced using an Applied Biosystems 373A automated sequencer.

Tissue Expression of PEX mRNA

PEX expression was examined in normal human tissues and in the Saos-2 human osteoblastic osteosarcoma cell line, by RT-PCR using oligonucleotides *PEX*-4 (5'-CTGGAT-CCTTGGTGGTCTAC-3' SEQ ID NO:8) and *PEX*-5 (5'-CACTGTGCAACTGTCTCAG-3' SEQ ID NO:9) as sense and antisense primers (2398 and 2895 are the nucleotide positions of the 5' end of these primers designed from the full-length human *PEX* cDNA). Semiquantitative PCR analysis for *PEX* expression in human tissues was performed as previously described, following normalization for *GAPDH* message in all samples containing *PEX* transcripts.

Northern-blot Analysis

Total RNA was obtained from Tumor I and human Saos-2 osteosarcoma cells using the RNeasy Total RNA kit (Qiagen) and oligo(dT)-purified poly(A)⁺ RNA was isolated from Saos-2 total RNA using standard procedures. Twenty micrograms of Tumor I total RNA and 20 µg of Saos-2 poly(A)⁺ RNA were fractionated on 1% denaturing agarose gel, and transferred to nylon membrane (Hybond N⁺, Amersham). Hybridization was performed with

32p-labeled full-length human PEX cDNA (3.1 kb) in 7 mM Tris-HCl, 50% formamide, 10% dextran sulfate, 4 X SSC, 2 x Denhardt's solution and heat-denatured salmon sperm DNA (100 µg/ml). The blot was washed in 0.1 X SSC, 0.1% SDS for 20 min at 50°C, and subjected to autoradiography for 4 days.

In Vitro Transcription, Translation, and Analysis of Products

Plasmid pPEX was linearized with NotI and sense RNA strand was transcribed using T7 RNA polymerase. Translation reactions in rabbit reticulocyte lysate were performed in the presence of [³H]leucine according to the manufacturer's recommendations (Promega) with or without canine pancreas microsomal membranes. Products were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 8%). Autoradiography was performed after treating the gel with EN³HANCE (Dupont NEN), as previously described.

Generation of myc-tagged PEX, Transfection in COS-7 Cells, and Triton X-114 Extraction

Plasmid pPEX-myc was generated by PCR amplification of PEX cDNA using oligonucleotide PEXMyc1 as the sense primer (5'-TTGGATGTCAACGCCTCG -3' SEQ ID NO:10, 519 is the nucleotide position of the 5' end of this primer designed from the cloned human PEX cDNA) and PEXMyc2 as the antisense (5'-CTACCACAATCTACAGTTGTT-CAGGTCCTCTTCGCTAATCAGCTTTTGTTCATAGAGTCCATGCCTCTG-3' SEQ ID NO:11) primer. The latter encodes the human c-myc tag sequences (underlined) and PEX sequences corresponding to the carboxyl terminal of the mature protein (⁷⁴²RGMDSMEQKLISEEDLNNCRLW*). Following PCR, the amplified fragment was ligated to the pPCR II vector, excised by digestion with KpnI/NotI and inserted into the corresponding sites in the polylinker region of pCDNA3. The in-frame fusion protein was verified by DNA sequencing.

COS-7 cells maintained in Dulbecco's modified Eagle's medium (DMEM, 4,500 mg/L glucose with L-glutamine; JRH Biosciences, Lenexa, KS) supplemented with 10% fetal calf serum (FCS; GIBCO) and antibiotics (pen/strep) were plated at a density of 3×10^5 cells/well in 6-well cluster plates 24 h prior to transfection. Cells were washed with twice with PBS and incubated with 2 μ g of pPEX-myc plasmid DNA in 1 ml of DMEM containing 0.1% BSA, and DEAE-dextran (Pharmacia LKB) for 3.5 h at 37°C. Following incubation, the transfection medium was aspirated, the cells were shocked with 10% DMSO in PBS for 2 min, and then cultured in DMEM with 10% calf serum at 37°C for 48 h. Triton X-114 extraction were performed on cultured cells expressing myc-tagged PEX as described. The samples were then analyzed by immunoblotting using the 9E10 anti-myc monoclonal antibody.

Stable Transfection of A293 Cells and Immunofluorescence

A293 cells maintained in DMEM with 10% FCS were transfected with the pPEX-myc plasmid by electroporation and selection initiated using G418 (600 mg/ml for 14 days and then decreased to 400 mg/ml). Populations of stably transfected cells were recovered at the end of the selection period. For myc-tagged PEX indirect immunofluorescence, stably transfected cells plated on gelatin-coated coverslips were washed twice with PBS, fixed in 4% paraformaldehyde and in some experiments permeabilized with 0.5% Triton X-100. Cells were blocked with 10% FCS in DMEM for 30 min, washed and incubated for 1 hr at 37°C with the 9E10 anti-myc monoclonal antibody (1:500 dilution). Cells were subsequently washed and incubated in turn with fluorescein-conjugated sheep anti-mouse secondary antibody (1:250 dilution). Coverslips were rinsed extensively with PBS, mounted in medium (glycerol:Tris; 1:1) containing 2.5%

1,4-diazabicyclo-(2,2,2) octane (Sigma) and examined with fluorescent microscopy using appropriate filters.

Assay for membrane-bound endopeptidase activity

COS-7 cells transiently transfected with pCDNA3
5 vector alone, with vector containing human NEP cDNA
(generous gift of P. Crine, Université de Montréal), or
with pPEX plasmid, were washed and scraped in PBS. Fol-
lowing brief centrifugation, the cell pellets were
resuspended in 50 mM Tris-HCl, pH 7.4 and disrupted by
10 sonication. Homogenates were fractionated by sequential
centrifugation at 1,000 x g for 10 min and then at
100,000 x g for 60 min. The final precipitate was
washed with 50 mM Tris-HCl, pH 7.4, resuspended in the
same buffer, and assayed for endopeptidase activity.
15 The protein concentration in membrane fractions was
determined by the method of Bradford with bovine serum
albumin as standard.

[D-Ala²,Leu⁵] enkephalin (500 µM) was incubated
with COS cell membrane preparations (~60 µg of protein)
20 in 100 mM Tris-HCl, pH 7.0, at 37°C for 30 min (final
volume 30 µl). The reaction was terminated by the addi-
tion of 100 µl 0.1% TFA (v/v). Production of Tyr-D-Ala-
Gly was monitored using reversed-phase HPLC (Bondpak C-
18 reverse phase column, Waters) with a U.V. detector
25 set at 214 nm. A linear solvent gradient of 0% B to 40%
B in 60 min was used with a flow rate of 1.5 ml/min
(mobile phase A=0.1% TFA (v/v); mobile phase B=80% ace-
tonitrile/0.1% TFA). Tyr-D-Ala-Gly was identified by
co-chromatography with marker synthetic peptide. For
30 assessing PEX endopeptidase activity, 10 µg of PTH [1-
38] and PTH [1-34] peptides (Peninsula Laboratories;
Belmont, CA) were added to the membrane preparations.
For HPLC analysis of hydrolysis products, a linear sol-
vent gradient of 0% to 50% solution B was used at a

rate of 1.5 ml/min. MALDI-TOF mass spectrometry was performed on specific peptide fragments.

RESULTS

Cloning of Human PEX cDNA

5 At the initiation of these studies, *PEX* expres-
sion had been reported in minute amounts only in leuko-
cytes and fetal brain. We postulated that in states of
hypophosphatemia *PEX* expression may be increased and
therefore opted to use the OHO tumor as a tissue source
10 that may express considerably more *PEX*. Tissues
obtained from two tumors associated with OHO were used
to obtain total RNA and analysis for *PEX* mRNA expres-
sion was assessed by RT-PCR. As shown in Fig.1, *PEX*
transcripts were readily amplified from both tumor sam-
15 ples demonstrating the expected 509 bp fragment pre-
dicted from the published partial human *PEX* sequence
(The HYP Consortium (1995) *Nature Genetics* **11**, 130-
136). Total RNA extracted from two tumors associated
with OHO was reverse transcribed and amplified by PCR
20 (35 cycles) using human *PEX*-specific primers, *PEX*-1 and
PEX-2, designed from the published human sequence. The
expected 509 bp amplified fragment was obtained from
both tumor samples. Control, no cDNA added to the
amplification reaction, i.e. negative control; Marker,
25 Φ 174 DNA digested with HaeIII restriction endonuclease.

The cloning of the 3' end of *PEX* transcript was
performed by rapid amplification of the 3' end of the
cDNA (3' RACE), while the 5' of the cDNA was amplified
by anchored PCR, as described in Experimental Proce-
30 dures. Fig. 2A shows the nucleotide and predicted amino
acid sequence of the full-length human *PEX* cDNA cloned
from tumor tissues. Nucleotide and deduced amino acid
sequence of tumor-derived human *PEX* cDNA (Fig. 2A). The
numbering begins at the 5' end nucleotide as determined
35 by anchored PCR. Amino acids are given below each codon

using the single letter code. The putative start codon is indicated as /1 along with the deduced amino acid translation. Two stop codons preceding the predicted initiation ATG are in bold type. Asterisk (*) indicates an in-frame stop codon, while a large asterisk (*) denotes the putative prenylation site. A potential polyadenylation signal in the 3' untranslated region is underlined. Nine potential N-glycosylation sites are boxed. The sequence has been assigned GenBank accession No. (U82970).

The composite cDNA reveals a single open reading frame encoding a protein of 749 amino acids which displays homology (34.2% identity, 70% similarity) to human neprilysin (NEP; EC 3.4.24.11), and other members of the membrane-bound metalloendopeptidase family encompassing endothelin-converting enzyme-1 (ECE-1; 66% similarity) and the Kell antigen (60% similarity), suggesting that PEX is a novel member of this family of neutral endopeptidases, as previously suggested (The HYP Consortium (1995) *Nature Genetics* **11**, 130-136). Like the other members, PEX is a likely a glycoprotein with eight potential N-glycosylation sites and 10 cysteine residues that may be important for the proper folding and hence native conformation of the protein.

The ATG codon at position 604 was assigned as the initiator methionine since it is preceded by two in-frame TGA termination codons 36 and 63 basepairs upstream and conforms favorably to the Kozak consensus for vertebrate initiation of translation. The cloned cDNA identifies the first 3 and the last 108 amino acids of the predicted PEX gene product in addition to the published partial sequence. These additional amino acids comprise residues such as E⁶⁴² and H⁷¹⁰ that are shared by NEP, and may be critical for the formation of the active site of the protein and hence its enzymatic

activity. Three amino acid residues predicted from our cDNA clone differ from the published partial human *PEX* sequence, D363A (GAC to GCC), R403W (AGG to TGG), and A641G (GCG to GGA). To confirm that these alterations did not arise because of PCR errors, *PEX* sequences were amplified from Saos-2 human osteosarcoma cells (see below) and sequenced. In addition, the same alterations were subsequently described in the murine *PEX* cDNA, suggesting possible cloning artifacts in the published partial human *PEX* sequence. Our cloned sequences also encompass 603 nucleotides of the 5' untranslated region, and 276 nucleotides of the 3' untranslated region, including the canonical polyadenylation signal AATAAA, 19 nt upstream of the poly(A) tract. The human and the published mouse *PEX* cDNA sequences share extensive homology within the protein coding region (96% identity) as well as in the 5' and 3' non coding regions.

TMpred analysis of the human *PEX* sequence predicts that the protein has no apparent N-terminal signal sequence but has a single membrane-spanning helical domain comprising amino acid residues 21-39 (Fig. 2C). TMpred analysis of the *PEX* sequence showing a single membrane-spanning domain encompassing amino acid residues 21-39 (arrowhead). Numbers on the horizontal axis refer to the amino acid sequence. Amino acid homology between *PEX* and human NEP cDNA (Fig. 2B). Sequence comparison was performed using the LALIGN program.

This predicts its transmembrane topology to be that of a type II integral membrane protein with a 20-residue N-terminal cytoplasmic tail and a C-terminal of 700 amino acid residues containing the catalytic domain in the extracellular compartment. Unexpectedly, a CXXX box motif comprising amino acid residues 746CRLW was also identified at the carboxyl terminus of *PEX*. This

motif may serve as a site for prenylation, a post-translational lipid modification involved in a number of processes including facilitating membrane attachment, targeting of proteins to specific subcellular membrane compartments, promoting protein-protein interactions and regulating protein function.

Tissue Expression of PEX mRNA

We next examined PEX expression in a number of fetal and adult tissues and compared the level of expression to OHO tumor RNA using semi-quantitative RT-PCR (Fig. 3). Quantitative RT-PCR amplification of the PEX transcripts from total RNA prepared from human tissues and OHO-associated tumor. Relative expression levels for the PEX transcript were measured by quantifying PEX product in reversed-transcribed RNA samples that have been previously normalized for GAPDH levels. The specific primers used were as follows: for PEX, the forward primer was PEX-4 and the reverse primer PEX-5; for GAPDH, the primers were as previously described. PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. Control, negative control; Marker, Φ 174 DNA digested with HaeIII restriction endonuclease. Below, shown are the relative levels of PEX transcripts in various human tissues compared to those in the tumor.

PEX transcripts were expressed in human fetal calvarium and to a lesser degree in fetal kidney and skeletal muscle while no expression was apparent in fetal liver. PEX expression was also observed in the human osteoblastic osteosarcoma cell line, Saos-2. In adult tissues, PEX mRNA was identified in kidney, but not in liver, or endomyocardium. Recent studies have also reported PEX expression in human fetal bone, skeletal muscle, and liver as well as fetal and adult ovary and lung (Beck, L. et al. (1997) *J. Clin. Invest.*

99, 1200-1209; Grief, M. et al. (1997) *Biochem. Biophys. Res. Commun.* **231**, 635-639). Analysis following normalization for *GAPDH* message in all tissues containing *PEX* transcript disclosed that bone *PEX* expression is 2-10 fold higher than in other normal tissues examined. In comparison, OHO tumor *PEX* expression was twice the levels observed in fetal calvarium, consistent with its relative "overabundance" in these tissues.

Northern Blot Analysis

To determine the size of the full-length *PEX* transcript, we isolated total RNA from tumor I (quantity of available tissue was insufficient for poly(A)⁺ RNA extraction) and poly(A)⁺ RNA from human Saos-2 osteosarcoma cells. This cell line was used since it is readily available and successful amplification of *PEX* sequences has been performed by RT-PCR (see above). Aliquots (20 µg of each) were examined by Northern-blot analysis using the cloned human *PEX* cDNA as probe. A single transcript of approximately 6.5 kb was readily detected only in the Saos-2-derived poly(A)⁺ sample and contrasts with the predicted size of the cloned sequence of 3.1 kb (Fig. 4). Approximately 20 µg of poly(A)⁺ RNA prepared from Saos-2 cells and 20 µg of total RNA prepared from tumor I tissue were resolved on 1% agarose gel containing formaldehyde and then transferred to a nylon membrane. Following hybridization with radiolabeled *PEX* cDNA, the blot was washed and the signal detected by autoradiography. A transcript of ~6.5 kb was observed only in the lane containing Saos-2 poly(A)⁺ RNA. There is suggestion of an additional band corresponding to a transcript of ~3.8 kb. Arrows indicate the position of the 28S (approx. 4.8 kb) and 18S (approx. 1.8 kb) ribosomal RNA.

This finding would therefore predict a ~4 kb 5' untranslated region for *PEX* cDNA, consistent with pub-

lished data from Northern blot analysis of *PEX* expression in mouse calvaria (Du, L. et al. (1996) *Genomics* 36, 22-28). A less well defined band was also detected in the Saos-2 sample corresponding to a potential transcript of ~3.8 kb, although the nature of this transcript remains unclear. Northern analysis of total RNA samples from tumor I and Saos-2 cells (results not shown) did not reveal any signal for *PEX*, consistent with the relatively low expression levels of the *PEX* transcript, previously described (The HYP Consortium (1995) *Nature Genetics* 11, 130-136; Beck, L. et al. (1997) *J. Clin. Invest.* 99, 1200-1209; Grieff, M. et al. (1997) *Biochem. Biophys. Res. Commun.* 231, 635-639). This finding contrasts sharply with *PEX* expression levels demonstrated in murine calvaria and cultured osteoblasts (Du, L. et al. (1996) *Genomics* 36, 22-28) and may reflect tissue and species differences.

In vitro translation of PEX cRNA

In vitro translation studies using full-length human *PEX* cRNA were performed in the rabbit reticulocyte lysate cell-free system. In the absence of microsomal membranes, *PEX* cRNA was translated into an ~86 kD protein, as predicted from the cloned cDNA sequence (Fig. 5). Plasmid p*PEX* was linearized and sense RNA strand transcribed using T7 RNA polymerase. Translation of *PEX* cRNA was performed using rabbit reticulocyte lysate in the absence (minus) and presence (plus) of canine pancreas rough microsomes. Products were electrophoresed in a SDS-polyacrylamide gel (10%) and visualized by autoradiography. Arrowhead in lane 2 indicates full-length human *PEX* protein. The addition of microsomal membranes results in the appearance of higher molecular weight forms that likely represent glycosylated products.

Following addition of canine microsomal membranes to the translation mixture, products of higher molecular weight (~100 kD) became apparent, consistent with N-glycosylation of *PEX* at the eight potential glycosylation sites deduced from the predicted sequence.

PEX is a Cell Membrane-Associated Protein

Previous studies have established that NEP, ECE-1 and Kell blood group glycoprotein are integral membrane proteins. We have used extraction with the detergent Triton X-114 and immunofluorescent localization to examine whether *PEX* is also a membrane-associated protein. For identification of *PEX*, we generated a construct in which the carboxyl terminus sequences of *PEX* are modified by a human c-myc tag. The epitope tag was inserted immediately upstream of the putative prenylation motif so that any potential lipid modification of the *PEX* protein may proceed uninterrupted.

Triton X-114 is a detergent that forms an aqueous solution at 4°C but separates into hydrophobic and aqueous phases when the temperature is raised to 30-37°C. This property has been used as an indicator of the hydrophobic nature of proteins, with integral membrane proteins partitioning exclusively in the detergent phase while highly hydrophilic proteins associate with the aqueous phase. Triton X-114 extracts from COS-7 cells transiently expressing *PEX* tagged with the c-myc epitope showed that *PEX* partitions nearly exclusively into the detergent phase (Fig. 6A). Extraction and partitioning of *PEX* expressed in COS-7 cells with Triton X-114 (Fig. 6A). Plasmid p*PEX*-myc was transiently transfected in COS-7 cells and 48 h later cells were extracted with Triton X-114. Whole cell extracts, as well as detergent and aqueous phases, were analyzed by SDS-PAGE and immunoblotted with an anti-myc monoclonal antibody. Right margin indicates $M_r \times 10^{-3}$.

This finding indicates that *PEX* is a membrane-associated protein and is consistent with the prediction from sequence analysis that it is an integral membrane protein.

5 To determine the subcellular localization of *PEX*, the distribution of recombinant protein expressed in stably transfected A293 cells was examined using immunofluorescence. When cells were fixed and permeabilized, myc-tagged *PEX* immunostaining was detected primarily on the cell surface, but in a number of cells
10 staining was also observed intracellularly, although no signal was observed in the nucleus (Fig. 6B). If permeabilization was omitted, staining was localized exclusively to the plasma membrane (Fig. 6C), while untransfected cells or cells transfected with vector alone
15 showed no immunofluorescent staining. Localization of *PEX* using indirect immunofluorescence in stably transfected A293 cells with (Fig. 6B) and without (Fig. 6C) permeabilization with Triton X-100, respectively.
20 Staining was carried out using the 9E10 anti-myc monoclonal antibody, followed by fluorescein-labeled secondary (sheep anti-mouse) antibody. Arrowheads indicate intracellular (B) and plasma membrane staining (C).

Since the myc-tag was inserted in the carboxyl end of *PEX*, these findings further corroborate the
25 sequence-based prediction that *PEX* is a type II integral membrane protein with its large C-terminal hydrophilic domain in the extracellular compartment.

Recombinant *PEX* protein has endopeptidase activity

30 The subcellular localization and sequence similarity between *PEX* and NEP strongly suggest that *PEX* functions as a membrane-bound metallopeptidase. However, no peptidase activity has been ascribed to *PEX*. As shown in Fig. 7A, when [D-Ala², Leu⁵] enkephalin,
35 used to assay for NEP activity, was incubated with cell

membrane preparations from vector-transfected COS cells or COS cells expressing equivalent amounts of recombinant human NEP or PEX proteins, as determined by Western blot analysis, production of Tyr-D-Ala-Gly from the substrate was evident only in NEP-expressing membrane preparations. Cell membrane preparations from vector transfected COS-7 cells (Fig. 7A) or from cells transiently expressing human NEP (Fig. 7B) or, human PEX cDNAs (Fig. 7C) were incubated in the presence of [D-Ala²,Leu⁵]enkephalin (500 μ M) and hydrolysis products were resolved by HPLC as described in the *Experimental Procedures* section. Tyr-D-Ala-Gly was identified by chromatography of synthetic marker peptide.

While the PEX sequence preserves two of the residues critical for catalytic activity of NEP (equivalent to E646 and H711), it lacks a residue equivalent to R102 shown to be crucial for the dipeptidylcarboxypeptidase activity of NEP. Therefore, unlike NEP, PEX has no dipeptidylcarboxypeptidase activity, but likely functions as an endopeptidase.

To examine recombinant human PEX for endopeptidase activity, cell membrane preparations from COS cells transiently expressing the protein were incubated with human PTH [1-38] or PTH [1-34] and the cleavage products were analyzed by reverse-phase high pressure liquid chromatography (HPLC), as shown in Fig. 8. Human PTH [1-38] was incubated with cell membrane preparations from vector transfected COS-7 cells (Fig. 8A) or from cells transiently expressing human PEX and hydrolysis products were resolved by HPLC (Fig. 8B). Chromatographic profile of products arising from the hydrolysis of PTH [1-34] when incubated with cell membranes from COS-7 cells transiently expressing PEX (Fig. 8C). The novel product with a molecular weight of

630 likely corresponds to the terminal pentapeptide DVHNF of human PTH [1-34].

A parallel preparation from vector transfected COS cells did not appreciably cleave PTH [1-38]. However, in the presence of PEX, both PTH peptides were hydrolyzed in a highly reproducible pattern resulting in the formation of several peaks that absorb at 214 nm. Mass spectrometry of the peptide materials recovered from two product peaks gave m/z values of 861 and 630, respectively. While the former product was present in hydrolysates from both PTH [1-38] and PTH [1-34], the latter product was identified only in the PTH [1-34] hydrolysate and likely corresponds to the carboxyl terminal pentapeptide DVHNF of human PTH [1-34]. These findings provide the first direct evidence that recombinant PEX possesses endopeptidase activity and suggest that its substrate specificity may not be restricted to the putative phosphatonin but may include other circulating hormones or perhaps bone-derived autocrine/paracrine regulatory factors that regulate renal phosphate handling.

DISCUSSION

To gain insight into the role of PEX in normal physiology we have cloned the human full-length cDNA and studied its expression, subcellular localization, and peptidase activity. The cloned human PEX cDNA encodes a protein whose deduced amino acid sequence is identical to the published partial (The HYP Consortium (1995) *Nature Genetics* **11**, 130-136) and to the full-length sequences reported more recently (Beck, L. et al. (1997) *J. Clin. Invest.* **99**, 1200-1209; Grief, M. et al. (1997) *Biochem. Biophys. Res. Commun.* **231**, 635-639; Guo, R. and Quarles, L. D. (1997) *J. Bone Miner. Res.* **12**, 1009-1017). Its deduced topology is that of a type II integral membrane glycoprotein and in the pres-

ent study we have provided experimental evidence to support this prediction. We have shown that *PEX* is glycosylated in the presence of canine microsomal membranes and partitions exclusively in the detergent phase following extraction with Triton X-114, consistent with the prediction from sequence analysis that it is an integral membrane glycoprotein. Nevertheless, the observed hydrophobic nature of *PEX*, need not be attributed solely to it being an integral membrane protein.

Lipophilic modification is known to cause cell membrane association, presumably through hydrophobic interaction of the modifying group with the lipid bilayer. Signaled by the C-terminal tetrapeptide CRLW motif, post-translational attachment of isoprenoids via a thioether linkage to the cysteine residue would be sufficient to promote effective membrane association. Further studies will be necessary to determine if such lipid modification of *PEX* does indeed take place. Of interest, however, is the observation that a nonsense mutation within this motif (R747Stop) has been reported to cosegregate with HYP and is likely to be associated with an inactive *PEX* gene product. Finally, the localization of *PEX* expressed in A293 cells is also consistent with the protein being membrane-associated and corroborates the sequence-based prediction that *PEX* is a type II integral membrane protein with its large C-terminal hydrophilic domain in the extracellular compartment. While protein expression was detected mostly on the cell surface, in some cells the signal was also localized intracellularly. This localization of the expressed protein would indicate that a portion of *PEX* activity is located in a membrane-bound compartment, possibly the Golgi membranes. The Golgi localization described for ECE-1 activity in cultured endothelial cells is proposed to promote the efficient conversion

of big endothelin-1 because of the co-localization and concentration of enzyme and substrate through the constitutive secretory pathway. It is possible then, that in parallel fashion, the PEX enzyme mediates both intracellular and cell-surface conversions of its putative substrate.

The finding that wild-type PEX transcripts are expressed in relative overabundance in OHO tumors poses a question in trying to understand the pathophysiology of these disorders. That is, how do we reconcile the apparently disparate observations that overexpression of PEX in OHO and loss of function in HYP patients, both lead to similar derangement in phosphate homeostasis? One of the physiological functions of PEX may well be the inactivation of a factor that normally promotes renal phosphate excretion (Fig. 9). The diagrams indicate events proposed to occur at the level of the proximal renal tubule. A putative circulating phosphaturic hormone (PHa) interacts with its renal receptor (PR) and inhibits phosphate reabsorption across the renal brush border membrane (-|) by decreasing NaPi activity. Downward arrows indicate the degree of phosphate excretion. PEX expressed predominantly in extrarenal tissues modulates the levels of circulating PHa by converting it to its inactive form (PHi).

In patients with OHO, the hyperphosphaturia that characterizes the syndrome would be the consequence of unregulated and excessive elaboration of the phosphaturic factor by the tumor. The modestly elevated PEX levels that we have documented in these tumors may arise either in response to the severe hypophosphatemia or to the abnormally high levels of the active phosphaturic factor. Yet, the increased PEX expression may not be sufficient to accommodate the increased substrate load, resulting in abnormally high circulating levels of the

active phosphaturic hormone. The inactivation of PEX observed in HYP patients would similarly cause decreased turnover of this humoral phosphaturic factor and thereby lead to renal phosphate wasting.

5 This model is also consistent with the observation that the *Hyp* phenotype is neither corrected nor transferred following cross transplantation of kidneys in normal and *Hyp* mice. Thus, when *Hyp* mice are engrafted with a normal kidney, phosphaturia ensues since circulating levels of the phosphaturic agent are excessive. On the other hand, engraftment of mutant kidneys in normal mice will not affect renal tubular phosphate handling of the recipients since circulating levels of the phosphaturic substance will be normally regulated by the enzymatic activity of extrarenal wild-type PEX. Indeed, analysis of the tissue distribution of PEX mRNA by RT-PCR has confirmed its expression in extrarenal tissues and particularly bone. Our present findings and those of others (Du, L. et al. (1996) *Genomics* **36**, 22-28; Beck, L. et al. (1997) *J. Clin. Invest.* **99**, 1200-1209; Grieff, M. et al. (1997) *Biochem. Biophys. Res. Commun.* **231**, 635-639; Guo, R. and Quarles, L. D. (1997) *J. Bone Miner. Res.* **12**, 1009-1017) showing high levels of PEX expression in cells of the osteoblast lineage would be consistent with the intrinsic osteoblast defect postulated to exist in HYP patients and in *Hyp* mice.

Finally, although the deduced structure of PEX clearly suggests that it is a metalloprotease, no peptidase activity had been ascribed to the protein. The preservation of the catalytic glutamate and histidine residues (equivalent to E⁶⁴⁶ and H⁷¹¹ of NEP; Fig. 2B) would argue for such an activity. In addition, the wide range of PEX mutations in HYP patients that align with regions required for protease activity in NEP suggests

that PEX also functions as a protease. Here, for the first time, we provide experimental evidence that recombinant PEX indeed functions as an endopeptidase. Unlike NEP, however, the protein does not possess dipeptidylcarboxypeptidase activity since it lacks a residue equivalent to R¹⁰² of NEP. Our unexpected observation that PEX effectively degrades PTH raises the question of whether circulating PTH is the putative phosphatonin. Although extracts from some OHO tumors have been reported to stimulate renal adenylate cyclase and this activity was inhibited by PTH antagonists, most studies have excluded PTH and PTH-related peptide (PTHrP) activity in OHO-associated tumors. Moreover, calcium homeostasis is generally preserved in patients with HYP. It is more likely, therefore, that the enzyme is rather promiscuous in its substrate specificity. PEX may indeed modulate PTH bioavailability and bioactivity, particularly at the level of the osteoblast, as well as the hormonal and paracrine/autocrine effects of factors produced by osteoblasts involved in regulating phosphate reabsorption and osteoblast maturation and mineralization. Although additional work will be required to clarify many of these issues, the availability of full-length human PEX cDNA now provides us with the opportunity to study the biology of PEX, identify its substrate(s), elucidate its role in pathological states characterized by dysregulated phosphate homeostasis, and determine its suitability as target for therapeutic intervention in the treatment of metabolic bone diseases.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following,

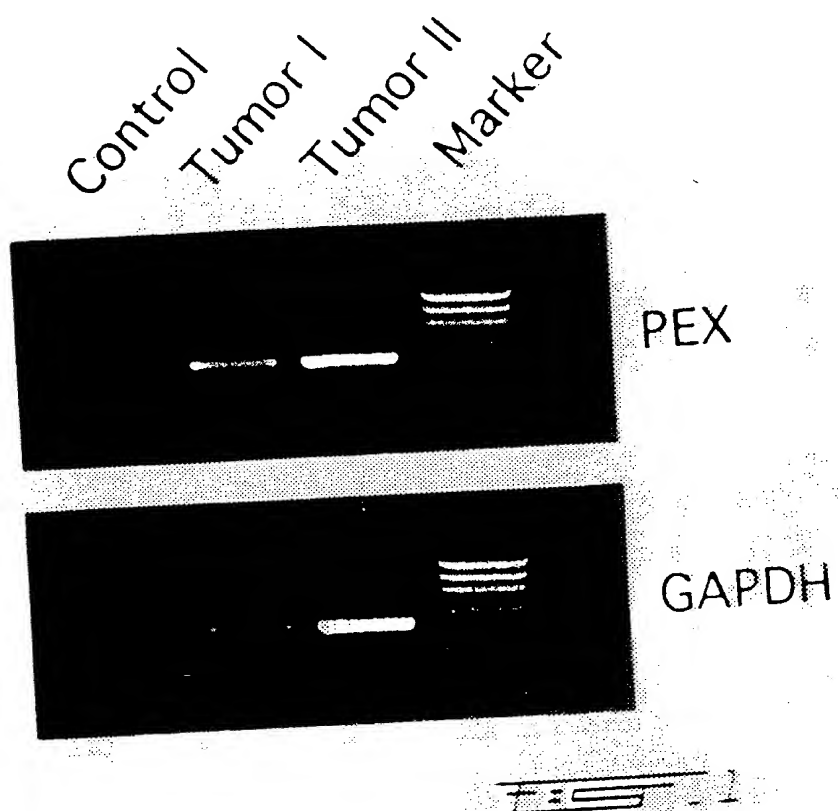
in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for the diagnosis of metabolic bone diseases in a patient, which comprises the step of determining the level of PTHrP in a biological sample of a patient wherein an alteration of PTHrP levels from that of a normal individual is indicative of metabolic bone diseases and/or metabolic bone diseases predisposition.
2. The method of claim 1, wherein said metabolic bone diseases is osteomalacia, osteoporosis, osteopetrosis or Paget's disease.
3. A method for the treatment of metabolic bone diseases, which comprises administering to a patient a compound for the modulation of PEX enzymatic activity.
4. The method of claim 3, wherein said metabolic bone diseases is osteomalacia, osteoporosis, osteopetrosis or Paget's disease.
5. Use of a compound for the modulation of PEX enzymatic activity for the manufacture of a medicament for treating metabolic bone diseases.
6. The use of claim 5, wherein said metabolic bone diseases is osteomalacia, osteoporosis, osteopetrosis or Paget's disease.
7. A method for the treatment of metabolic bone diseases, which comprises modulating PTH and PTHrP levels that regulate osteoblast activity in a patient to modulate bone breakdown and bone formation.
8. The method of claim 8, wherein said metabolic bone diseases is osteomalacia, osteoporosis, osteopetrosis or Paget's disease.
9. Use of modulation of PTH and PTHrP levels that regulate osteoblast activity for the treatment of metabolic bone diseases.

10. The use of claim 9, wherein said metabolic bone diseases is osteomalacia, osteoporosis, osteopetrosis or Paget's disease.
11. A non-human transgenic mammal to study the role of PEX in bone development and homeostasis, whose germ cells and somatic cells contain a PEX gene construct for expression of PEX in osteoblast consisting essentially of a recombinant PEX gene sequence under the control of a proximal promoter of a pro- α 1(I) collagen gene, the PEX gene construct being introduced into the mammal, or an ancestor of the mammal, at an embryonic stage.
12. The non-human mammal of claim 11, which is a mouse and the proximal promoter is murine pro- α 1(I) collagen gene.
13. The non-human mammal of claim 12, wherein said murine pro- α 1(I) collagen gene is a 2.3 kb fragment thereof.

1/18



2/18

1 GAT CCA CTA GTA ACG GCC GCC AGT GTG GTG GAA TTC AAG GGA CTC ACA CAC TGA AAG AAT 31
 61 ATC TTT GAT GAA GAC AAT TCA GGC AAG CAG AAT GAT TCT TGC AAC AGA ATT ACA TGA TTA 91
 121 GAG ATC TTG AAG TGG GTC CGG TGA ATC CTG GCC ACC TAA CTT ATC ATG ATT TGG GGG 151
 ATT GAG ATC TTG AAG TGG GTC CGG TGA ATC CTG GCC ACC TAA CTT ATC ATG ATT TGG GGG 211
 181 TTC ACG AGA ATC CAG TTT TGA TAA AAC AAT TGT TTT CCT CCC CAA GTG ACT ATA 271
 AGT TTC ACG AGA ATC CAG TTT TGA TAA AAC AAT TGT TTT CCT CCC CAA GTG ACT ATA 271
 241 TTA AAT AGC TAA AAC ATC TGT TCA GCA ACA TAG TAA AAC ATA TAT ACT CGG AAC GCT 331
 CAT TTA AAT AGC TAA AAC ATC TGT TCA GCA ACA TAG TAA AAC ATA TAT ACT CGG AAC GCT 331
 301 GAG AAG AGC CTG CCA AAC AAG GAC TTT GCT GAG GGA GAG CAC CAA GAT AAA GCA ACA 391
 TGA GAG AAG AGC CTG CCA AAC AAG GAC TTT GCT GAG GGA GAG CAC CAA GAT AAA GCA ACA 391
 361 TTT GTT TTG TCT AGT CAG GGG GGA AAG CCA AGG CAA CCA ATA TTT TGG TTT TTA TAA 451
 CTG TTT GTT TTG TCT AGT CAG GGG GGA AAG CCA AGG CAA CCA ATA TTT TGG TTT TTA TAA 451
 421 TCA TTT GTG AAG AAT TAT TTG AGA AAG GGT TGG CGA GGG GAG ATT TCC TGA CGG CAG 511
 TTT TCA TTT GTG AAG AAT TAT TTG AGA AAG GGT TGG CGA GGG GAG ATT TCC TGA CGG CAG 511
 481 CTT AAG CTG TCC ATT AGT AGA AGA GCA AGA GAG CCT TGG ATG TCA ACG CCT CGC TCT 571
 TTT CTT AAG CTG TCC ATT AGT AGA AGA GCA AGA GAG CCT TGG ATG TCA ACG CCT CGC TCT 571
 541 GAC CAG CCA CCA AAC CAC GAA AAG TGA CTT TCT CGT GTG CTC TCT ACG GCC CTT 631/10
 TGA GAC CAG CCA CCA AAC CAC GAA AAG TGA CTT TCT CGT GTG CTC TCT ACG GCC CTT 631/10
 601 /1 GAA GCA GAA ACA GGG AGC AGC GTG GAG ACT GGA AAG AAG GCC AAC AGA GGC ACT 691/30
 CTG ATG GAA GCA GAA ACA GGG AGC AGC GTG GAG ACT GGA AAG AAG GCC AAC AGA GGC ACT 691/30
 M E A E T G S S V E T G K K A N R G T
 661/20 GAT CCA CTA GTA ACG GCC GCC AGT GTG GTG GAA TTC AAG GGA CTC ACA CAC TGA AAG AAT
 CGA ATT GCC CTG GTC GTG TTT GTC GGT GGC ACC CTA GTT CTG GGC ACG ATC CTC TTT CTA
 R I A L V V F V G G T L L V L G T I L F L
FIG. 2A (cont.)

721/40 GTG AGT CAA GGT CTC TTA AGT CTC CAA GCT AAA CAG GAG TAC TGC CTG AAG CCA GAA TGC
 V S Q G L L S L Q A K Q E Y C L K P E C
 751/50
 781/60 ATC GAA GCG GCT GCT GCC ATC TTA AGT AAA GTA AAT CTG TCT GTG GAT CCT TGT GAT AAT
 I E A A A I L S K V N L S V D P C D N
 811/70
 841/90 TTC TTC CCG TTC GCT TGT GAT GGC TGG ATA AGC AAT AAT CCA ATT CCC GAA GAT ATG CCA
 F F R F A C D G W I S N P I P E D M P
 901/100 AGC TAT GGG GTT TAT CCT TGG CTG AGA CAT AAT GTT GAC CTC AAG TTG AAG GAA CTT TTG
 S Y G V Y P W L R H N V D L K E L L
 931/110
 961/120 GAG AAA TCA ATC AGT AGA AGG CGG GAC ACC GAA GCC ATA CAG AAA GCC AAA ATC CTT TAT
 E K S I S R R D T E A I Q K A K I L Y
 1051/150
 1021/140 TCA TCC TGC ATG AAT GAG AAA GCG ATT GAA AAA GCA GAT GCC AAG CCA CTG CTA CAC ATC
 S S C M N E K A I E K A D A K P L L H I
 1111/170
 1081/160 CTA CGG CAT TCA CCT TTC CGC TGG CCC GTG CTT GAA TCT AAT ATT GGC CCT GAA GGG GTT
 L R H S P F R W P V L E S N I G P E G V
 1171/190
 1141/180 TGG TCA GAG AGA AAG TTC AGC CTT CTG CAG ACA CTT GCA ACG TTT CGT GGT CAA TAC AGC
 W S E R K F S L L Q T L A T F R G Q Y S
 1231/210
 1201/200 AAT TCT GTG TTC ATC CGT TTG TAT GTG TCC CCT GAT GAC AAA GCA TCC AAT GAA CAT ATC
 N S V F I R L Y V S P D D K A S N E H I
 3/18
~~751/50~~ - 2A (cont.)

1261/220
 TTG AAG CTG GAC CAA GCA ACA CTC TCC CTG GCC GTG AGG GAA GAC TAC CTT GAT AAC AGT
 L K L D Q A T L S L A V R E D Y L D N S
 1321/240
 ACA GAA GCC AAG TCT TAT CGG GAT GCC CTT TAC AAG TTC ATG GTG GAT ACT GCC GTG CTT
 T E A K N S Y R D A L Y K F M V D T A V L
 1381/260
 TTA GGA GCT AAC AGT TCC AGA GCA GAG CAT GAA GAC ATG AAG TCA GTG CTC AGA TTG GAA ATT
 L G A N S S R A E H D M K S V L R L E I
 1441/280
 AAG ATA GCT GAG ATA ATG ATT CCA CAT GAA GAC CGA ACC AGC GAG GCC ATG TAC AAC AAA
 K I A E I M I P H E N R T S E A M Y N K
 1501/300
 ATG AAC ATT TCT GAA CTG AGT GCT ATG ATT CCC CAG TTC GAC TGG CTG GGC TAC ATC AAG
 M N I S E L S A M I P Q F D W L G Y I K
 1561/320
 AAG GTC ATT GAC ACC AGA CTC TAC CCC CAT CTG AAA GAC ATC AGC CCC TCC GAG AAT GTG
 K V I D T R L Y P H L K D I S P S E N V
 1621/340
 GTG GTC CGC GTC CCG CAG TAC TTT AAA GAT TTG TTT AGG ATA TTA GGG TCT GAG AGA AAG
 V V R V P Q Y F K D L F R I L G S E R K
 1681/360
 AAG ACC ATT GCC AAC TAT TTG GTG TGG AGA ATG GTT TAT TCC AGA ATT CCA AAC N L S
 K T I A N Y L V W R M V Y S R I P N L S
 1741/380
 AGG CGC TTT CAG TAT AGA TGG CTG GAA TTC TCA AGG GTA ATC CAG GGG ACC ACA ACT TTG
 R R F Q Y R W L E F S R V I Q G T T T L
 1291/230
 GCC GTG AGG GAA GAC TAC CTT GAT AAC AGT
 A V R E D Y L D N S
 1351/250
 TAC AAG TTC ATG GTG GAT ACT GCC GTG CTT
 Y K F M V D T A V L
 1411/270
 GAC ATG AAG TCA GTG CTC AGA TTG GAA ATT
 D M K S V L R L E I
 1471/290
 AAC CGA ACC AGC GAG GCC ATG TAC AAC AAA
N R T S E A M Y N K
 1531/310
 CCC CAG TTC GAC TGG CTG GGC TAC ATC AAG
 P Q F D W L G Y I K
 1591/330
 CTG AAA GAC ATC AGC CCC TCC GAG AAT GTG
 L K D I S P S E N V
 1651/350
 TTG TTT AGG ATA TTA GGG TCT GAG AGA AAG
 L F R I L G S E R K
 1711/370
 ATG GTT TAT TCC AGA ATT CCA AAC N L S
 M V Y S R I P N L S
 1771/390
 TCA AGG GTA ATC CAG GGG ACC ACA ACT TTG
 S R V I Q G T T T L

4/18

(cont.)

1801/400
CTG CCT CAA TGG GAC AAA TGT GTA AAC TTT ATT GAA AGT GCC CTC CCT TAT GTT GTT GGA
L P Q W D K C V N F I E S A L P Y V G

1831/410
1861/420
AAG ATG TTT GTA GAT GTG TAC TTC CAG GAA GAT AAG AAG GAA ATG ATG GAG GAA TTG GTT
K M F V D V Y F Q E D K K E M M E E L V

1891/430
1921/440
GAG GGC GTT CGC TGG GCC TTT ATT GAC ATG CTA GAG AAA GAA AAT GAG TGG ATG GAT GCA
E G V R W A F I D M L E K E N E W M D A

1951/450
2011/470
1981/460
GGA ACG AAA AGG AAA GCC AAA GAA AAG GCG AGA GCT GTT TTG GCA AAA GAT GGC TAT CCA
G T K R K A K E K A R A V L A K V G Y P

2041/480
2071/490
2101/500
GAG TTT ATA ATG AAT GAT ACT CAT GTT AAT GAA GAC CTC AAA GCT ATC AAG TTT TCA GAA
E F I M N D T H V N E D L K A I K F S E

2131/510
2161/520
2191/530
GCC GAC TAC TTT GGC AAC GTC CTA CAA ACT CGC AAG TAT TTA GCA CAG TCT GAT TTC TTC
A D Y F G N V L Q T R K Y L A A Q S D F F

2211/540
2251/550
2311/570
TTC TAC AGT GCA TCC ACC AAC CAG ATC CGA TTT CCA GCA GGA GAG CTC CAG AAG CCT TTC
F Y S A S T N Q I R F P A G E L Q K P F

2281/560
2311/570
TTT TGG GGA ACA GAA TAT CCT CGA TCT CTG AGT TAT GGT GCT ATA GGA GTA ATT GTC GGA
F W G G T E Y P R S L S Y G A I G V I V G

5/18

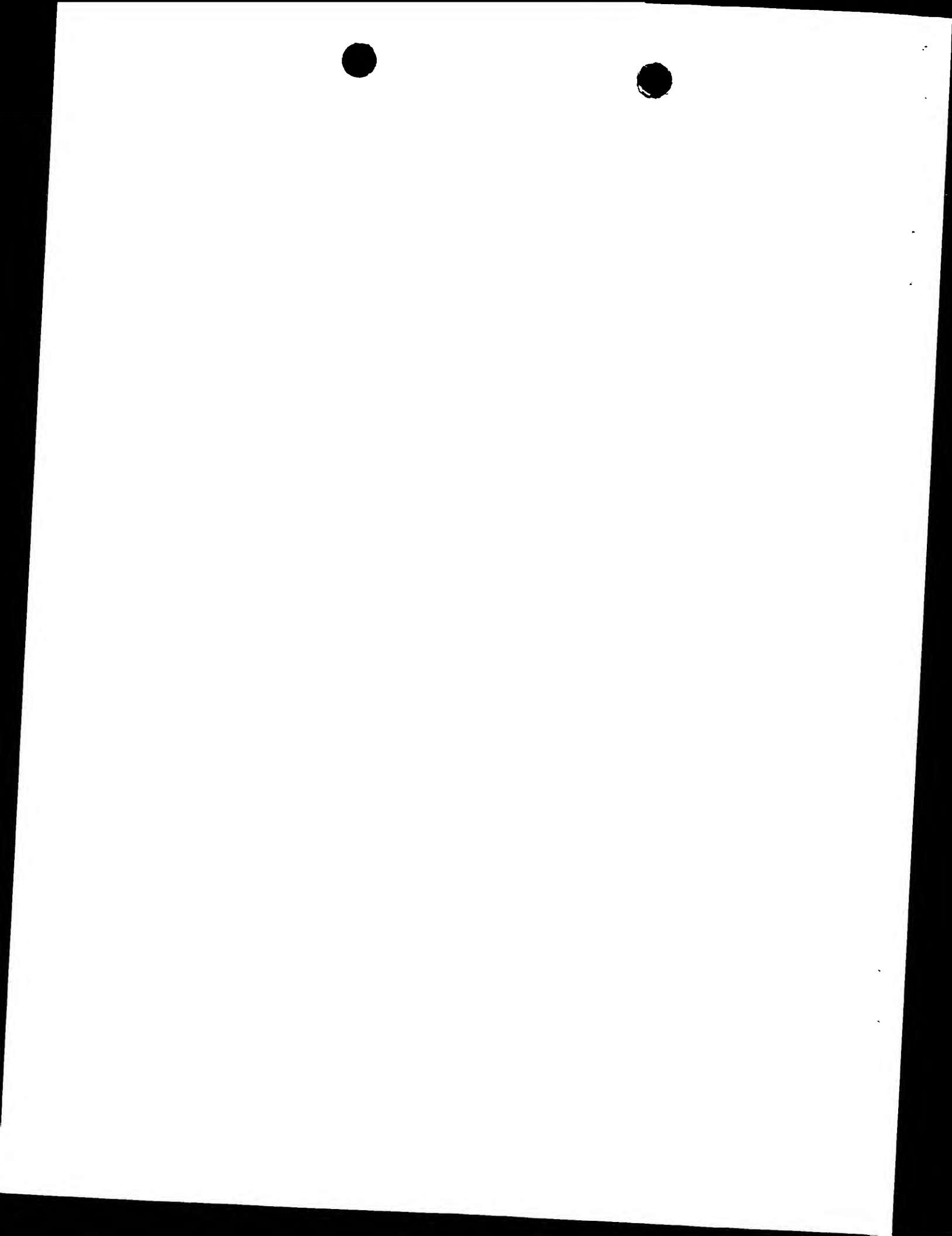
~~FILE~~ - 2A (cont.)

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7/18

2881 2991
 GAC AGT TGC ACA GTG CCA GCG GAG GCT GCA CTG AGC CTT CAT CGC CCA TTG CTT TAG GCC
 2941 2971
 TGG AGG AGC TTT CAT TTT TAG TGC ATT TTC ATT ATT TGG GTA GGT GAC CTG CTT GGA TCT
 3001 3031
 AGA CAG CAT CTG TTC AAA GTT GTA GGG CTT ATA AAG TGG AAT ATA AGA AGA ACT AAG TAT
 3051 3091
 GTT TCT TTA GAA AAT CAA ACC AAC AAA AAT AAA TCC CTA GGC TAC TTT TGT TAA AAA AAA
 3121
 AAA AAA AAA A

~~FILE~~ - 2A (cont.)



9/18

hPEX	AKSYRDALY-KFMVDTAVLLGANSRAEH----	DMKSVLRLEIKIAEIMIPHENRT-SEA
	250	290
hNEP	KEACTAYVDFMISVARLIRQEERLPIDENQLALEMNKVMELEKEIANATAKPEDRNDPML	
	240	290
hPEX	MYNKMNISEL-SAMIPQFDWLGYIK-KVIDTRLYPHLKDIPSENVVVRVPQYFKDLFRI	
	300	350
hNEP	LYNKMTLAQIQNNFSLKNGKPFWSLNFNEIMSTVNI SITNEEDVVVYAPEYLTCLKPI	
	300	350
hPEX	LGSEKKKTIANYLVMRMVYSRIPNLSRRFQYRWLEFSRVIOGTTLLPQWDKCVNFIESA	
	360	410
hNEP	LTKYSARDLQNLMSWRFIMDLVSSLSRTRYKESRNAFRKALYGTTSATWRRRCANYVNGN	
	360	410

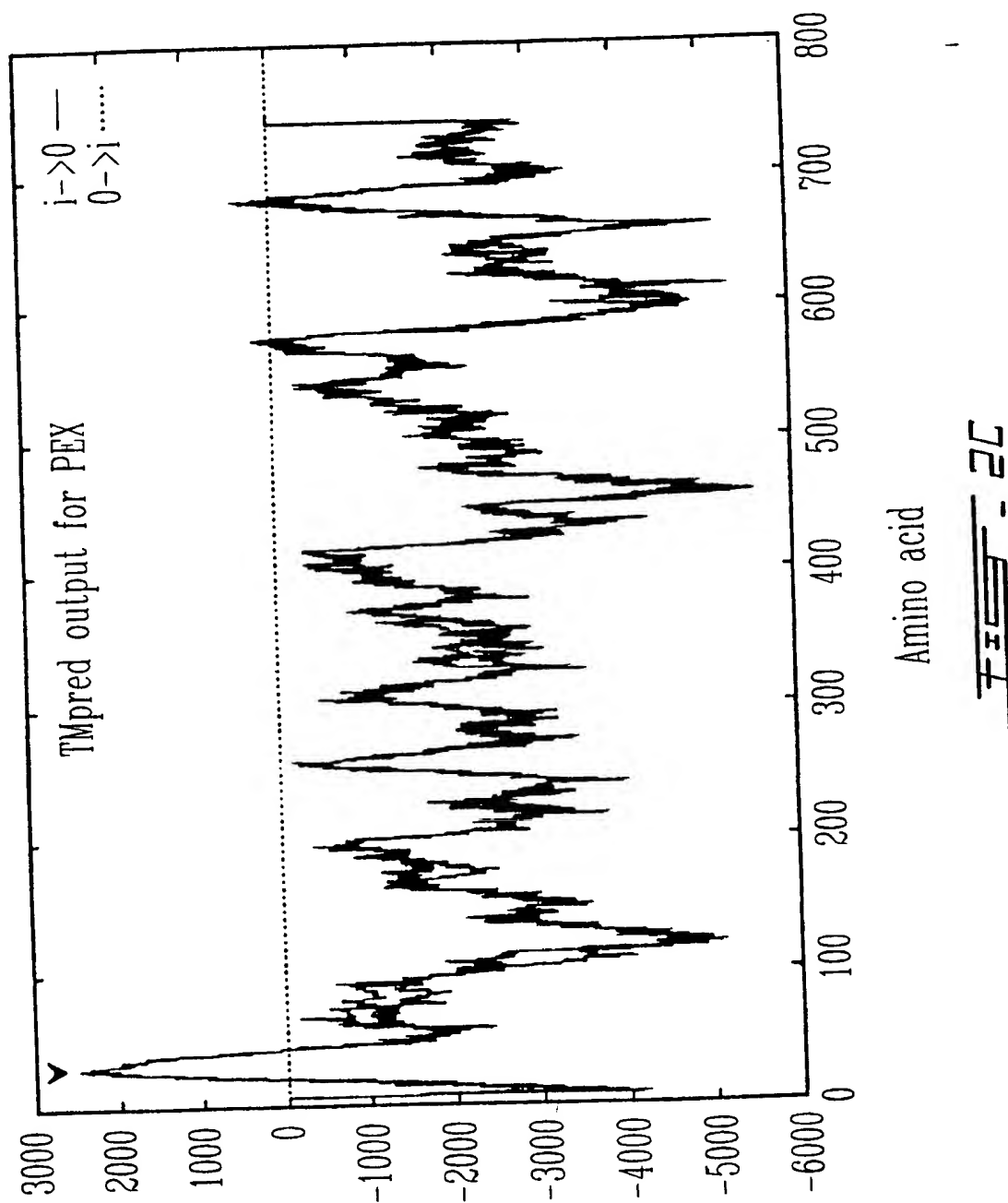
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11/18

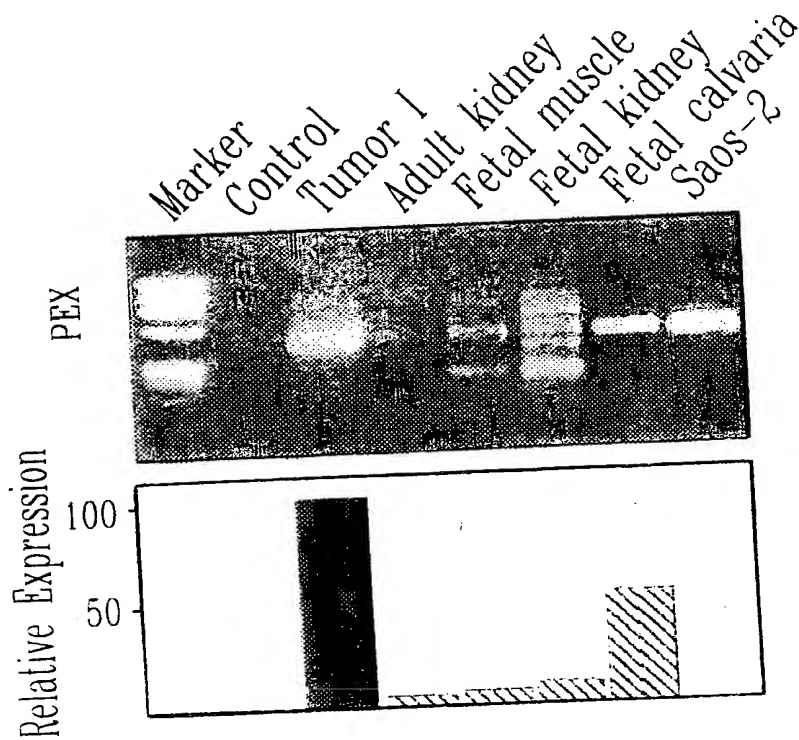
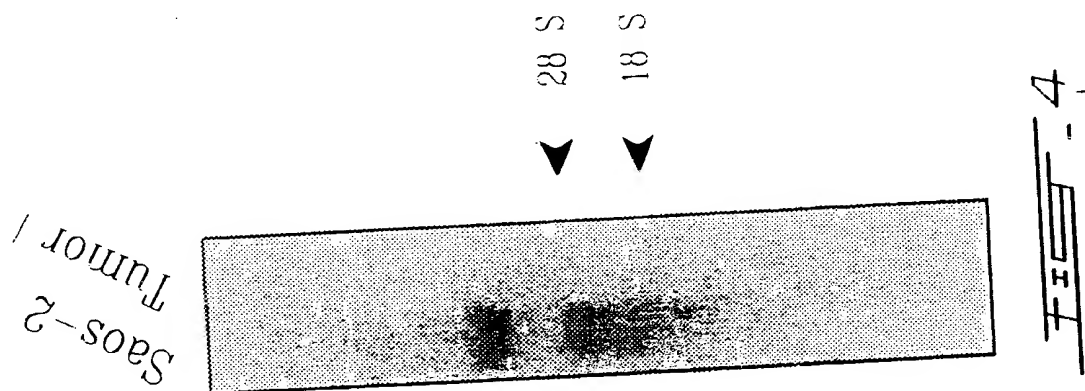
hPEX	600	610	620	630	640	650
	YDKNGNLDPW	STEESEKFK	EKTKCMIN	QYSNYWK	KAG-LNV	KGKRTLGENIADNGGLR
hNEP	600	610	620	630	640	650
	FNKDGDLVD	WTQQSAS	NFKEQSQ	CMVYQY	GNFSD	LAGGQHLNGINTLGENIADNGGLG
hPEX	660	670	680	690	700	710
	EAFRAYRKW	INDRRQGL	EEPLPGIT	TFTNNQL	FFLSYAH	VRCNSYRPEAAREQVQIGAHS
hNEP	660	670	680	690	700	710
	QAYRAYQ--	NYIKKN	GEEKLLP	GLDLN	HKQLFF	LNFAQVWC
hPEX	720	730	740			
	PPQFRVNG	AI	SNFEEFQ	KAFNC	PPN	STMTNRMGMDSCRLW
hNEP	720	730	740			
	PGNFRI	IGTLQNS	AEFSE	AFHCR	KNSYMN	PEKK-CRVW

FEF-2B (cont.)

12/18

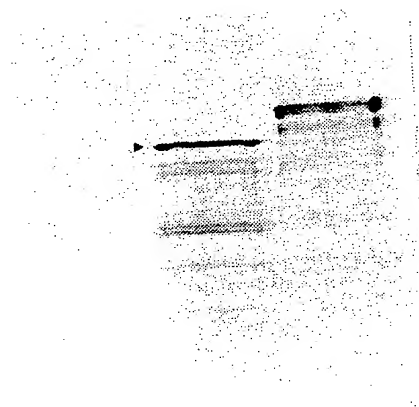


13/18



14/18

mRNA	-	+	+
Membranes	-	-	+



Mr (10³)

< 97.1

< 66.4

< 42.7

Fig. 5

15/18
TX114

A

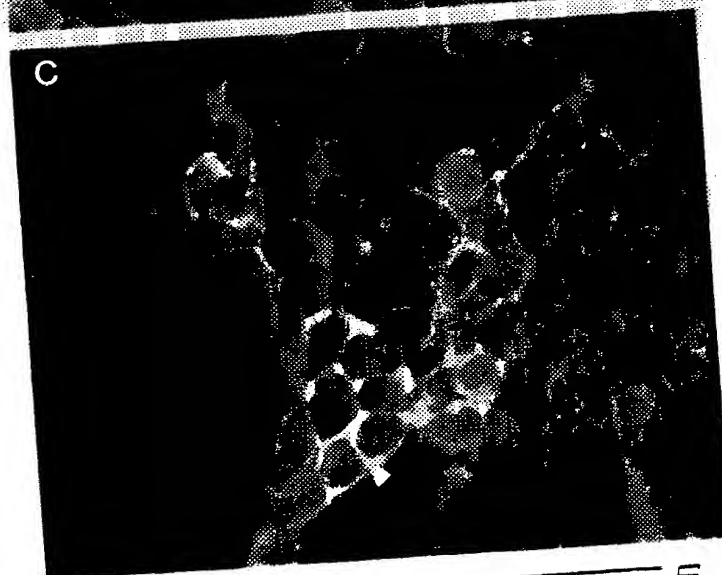
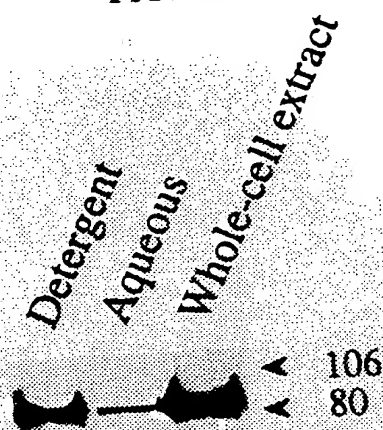
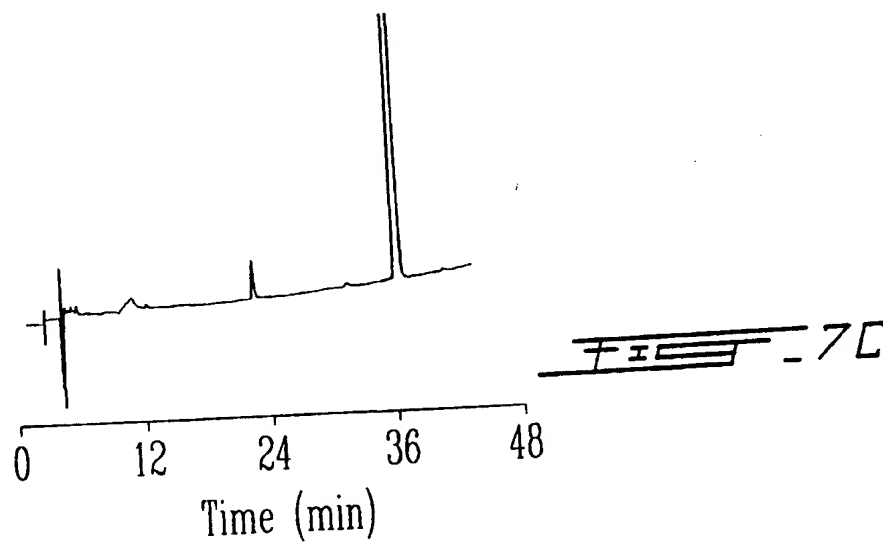
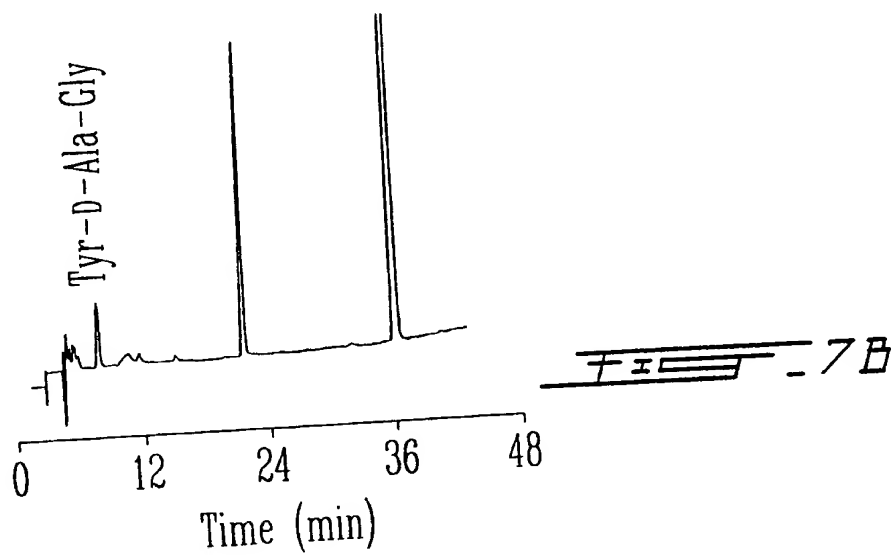
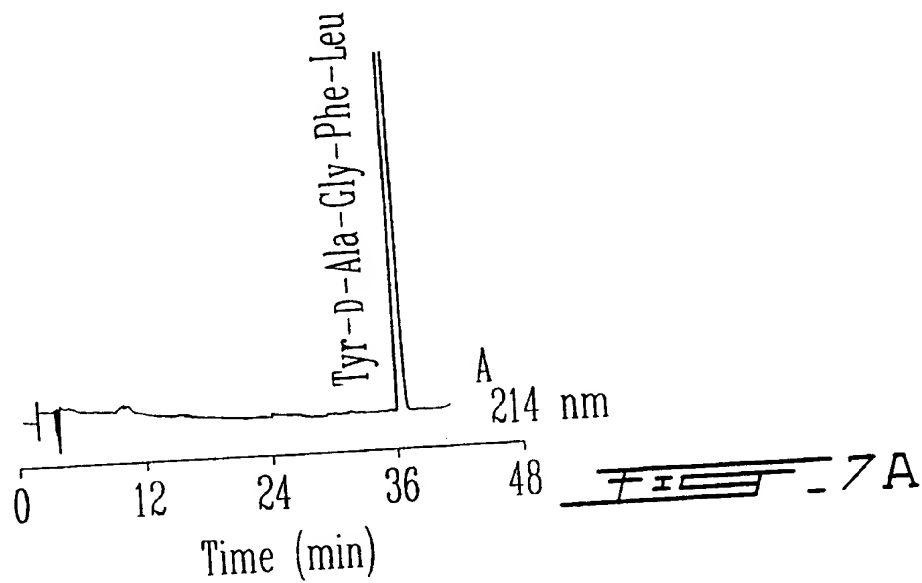
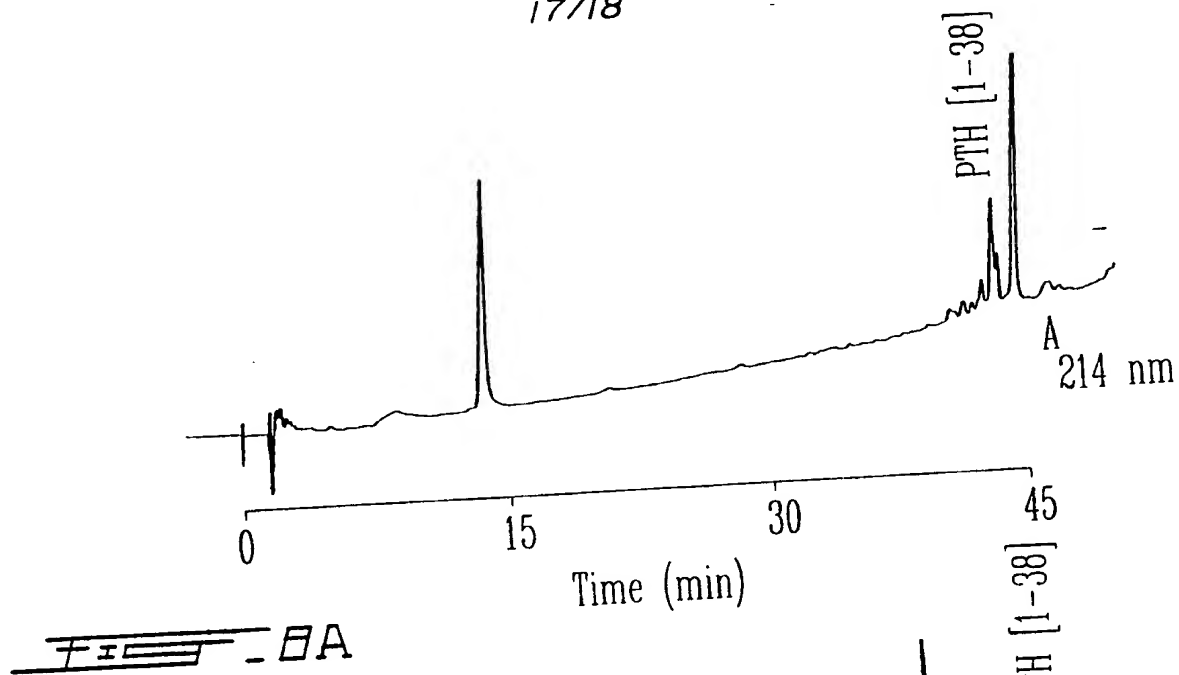


FIG. 6

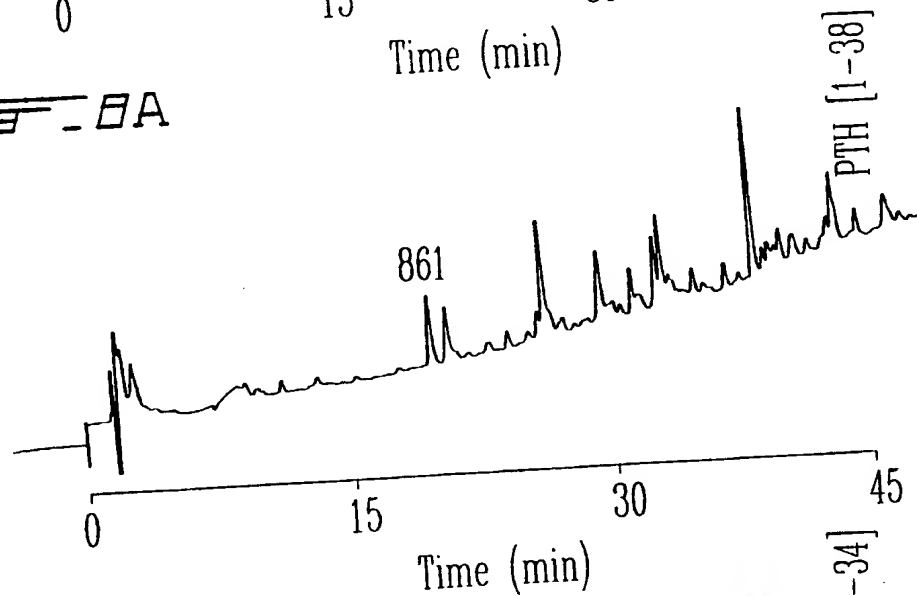
16/18



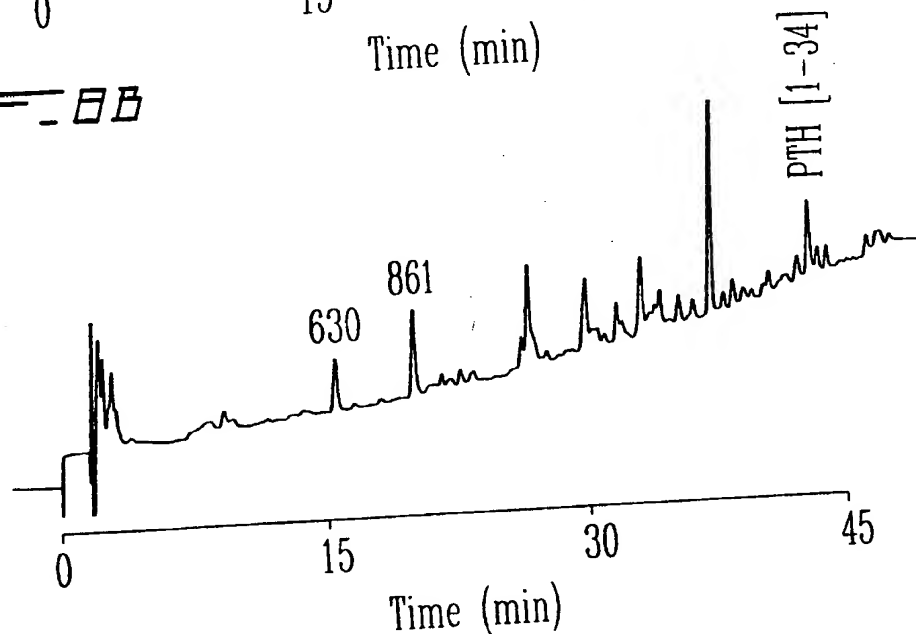
17/18



FIIS - BA



FIIS - BB



FIIS - BC

18/18

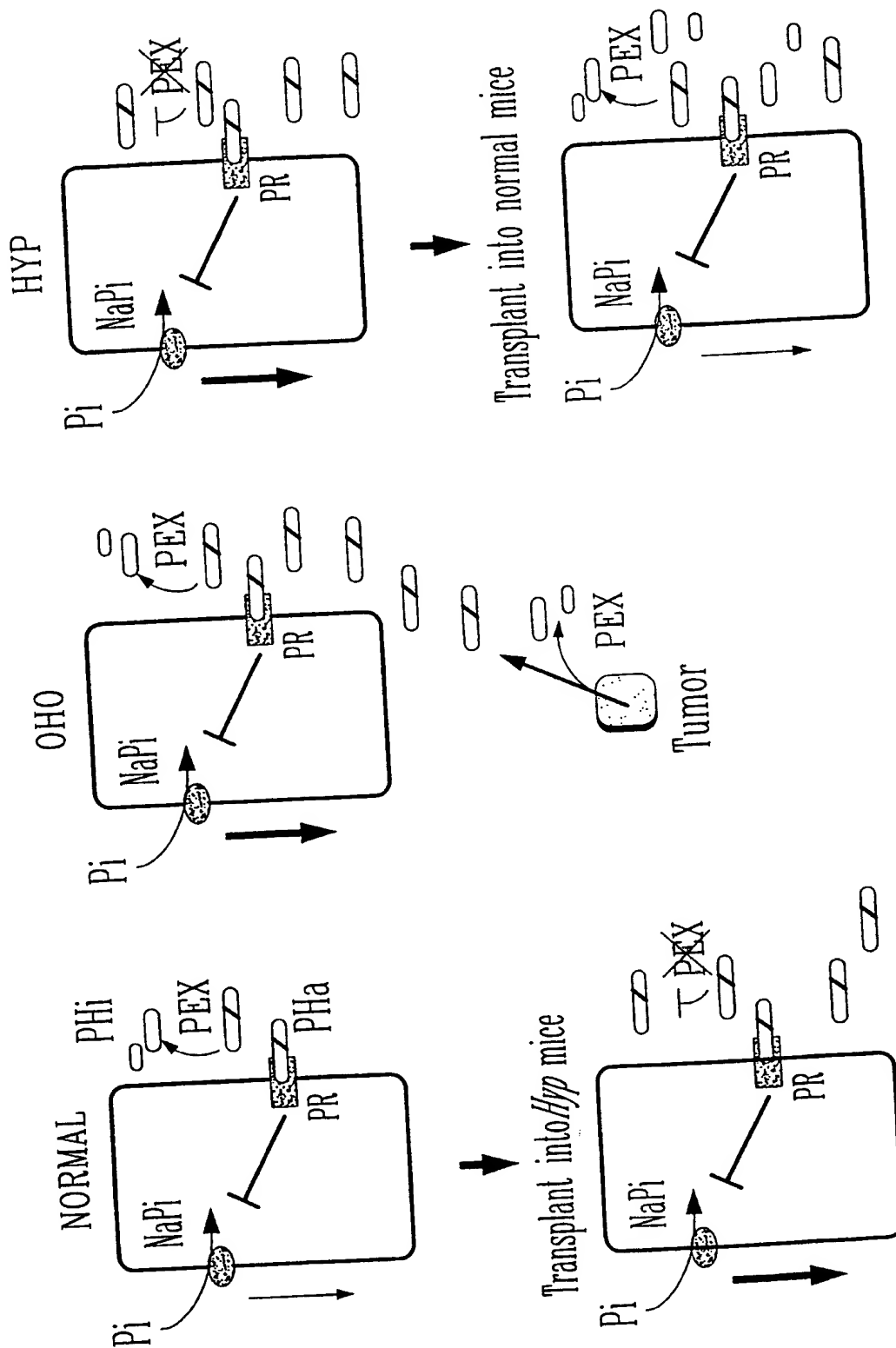
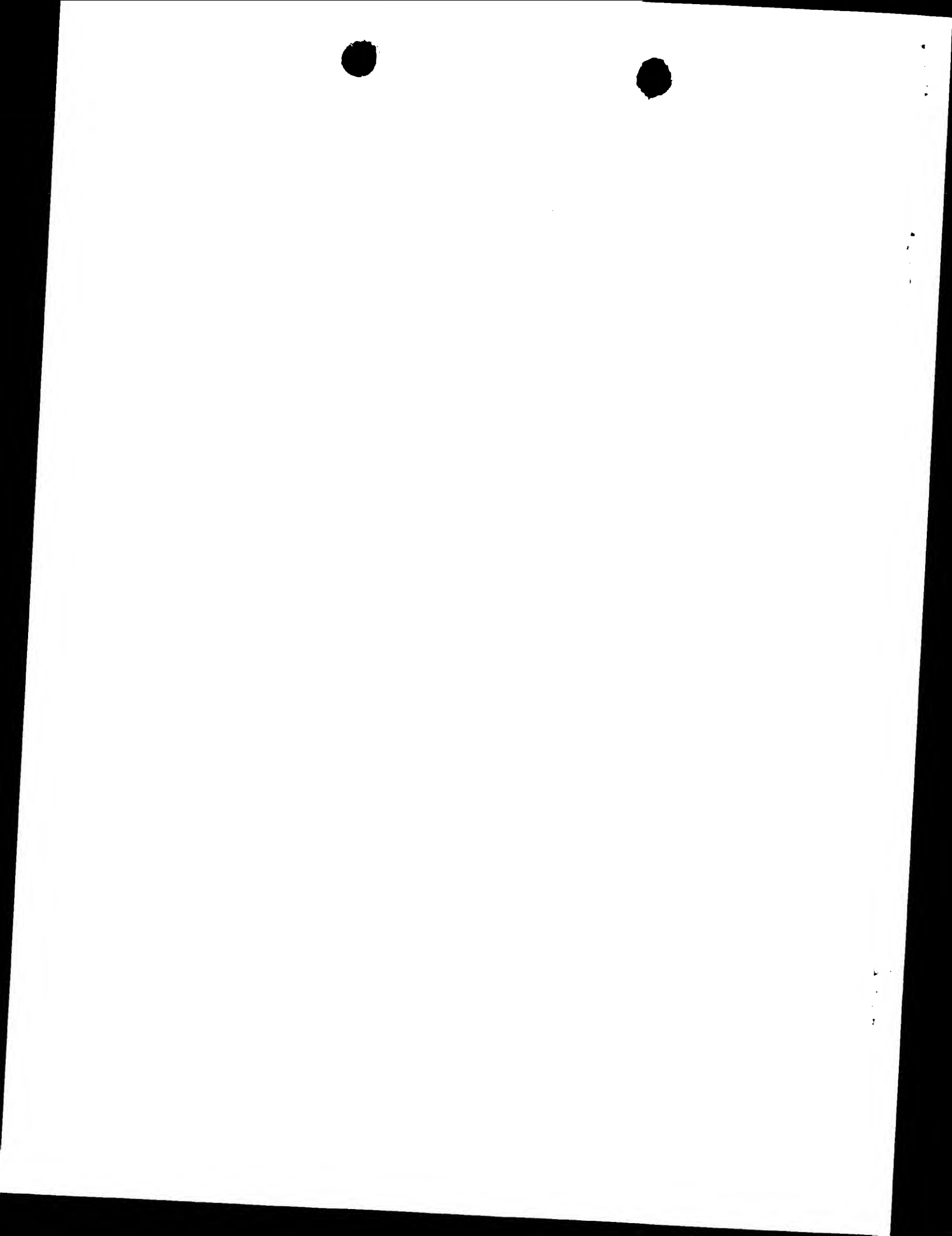


Fig. 9.



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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : A61K 38/48, A01K 67/027, A61P 19/00	A3	(11) International Publication Number: WO 00/18954 (43) International Publication Date: 6 April 2000 (06.04.00)
(21) International Application Number: PCT/CA99/00895 (22) International Filing Date: 27 September 1999 (27.09.99) (30) Priority Data: 2,245,903 28 September 1998 (28.09.98) CA (71) Applicant (for all designated States except US): MCGILL UNIVERSITY [CA/CA]; Office of Technology Transfer, 845 Sherbrooke Street West, Montréal, Québec H3A 2T5 (CA). (72) Inventors; and (75) Inventors/Applicants (for US only): KARAPLIS, Andrew, C. [CA/CA]; 95 Meaney, Kirkland, Québec H9J 3V6 (CA). GOLTZMAN, David [CA/CA]; 667 Belmont, Westmount, Québec H3Y 2W3 (CA). LIPMAN, Mark, L. [CA/CA]; 2258 Fulton Road, Town of Mount Royal, Québec H3R 2L4 (CA). HENDERSON, Janet, E. [CA/CA]; 70 Woseley, Montreal West, Québec H4X 1V7 (CA). (74) Agents: COTE, France et al.; Swabey Ogilvy Renault, Suite 1600, 1981 McGill College Avenue, Montréal, Québec H3A 2Y3 (CA).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> (88) Date of publication of the international search report: 6 July 2000 (06.07.00)
(54) Title: USE OF PEX IN THE TREATMENT OF METABOLIC BONE DISEASES (57) Abstract <p>The present invention relates to a method for the diagnosis of metabolic bone diseases in a patient, which comprises the step of determining the level of PTHrP in a biological sample of a patient wherein an alteration of PTHrP levels from that of a normal individual is indicative of metabolic bone diseases and/or metabolic bone diseases predisposition. The present invention also relates to a method for the diagnosis of metabolic bone diseases in a patient, which comprises the step of determining the level of PTHrP in a biological sample of a patient wherein an alteration of PTHrP levels from that of a normal individual is indicative of metabolic bone diseases and/or metabolic bone diseases predisposition.</p>		



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INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 99/00895

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/48 A01K67/027 A61P19/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
------------	--	-----------------------

X	LIPMAN MARK L ET AL: "Cloning of human PEX cDNA: Expression, subcellular localization, and endopeptidase activity." JOURNAL OF BIOLOGICAL CHEMISTRY MAY 29, 1998, vol. 273, no. 22, 29 May 1998 (1998-05-29), pages 13729-13737, XP002134628 ISSN: 0021-9258 the whole document	1-10
X	WO 98 10078 A (KARAPLIS A.C. ET AL.) 12 March 1998 (1998-03-12) page 11, line 15-30; claims 1-8 --- -/-	1-13

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

3 April 2000

Date of mailing of the international search report

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Moreau, J



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INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 99/00895

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		Relevant to claim No.
Category *	Citation of document, with indication, where appropriate, of the relevant passages	
A	<p>GUISE THERESA A ET AL: "Evidence for a causal role of parathyroid hormone-related protein in the pathogenesis of human breast cancer-mediated osteolysis." JOURNAL OF CLINICAL INVESTIGATION 1996, vol. 98, no. 7, 1996, pages 1544-1549, XP002134629 ISSN: 0021-9738 the whole document</p>	1-13
P,X	<p>SCHNEIDER HANS-GERHARD ET AL: "Parathyroid hormone-related protein mRNA and protein expressions in multiple myeloma: A case report." JOURNAL OF BONE AND MINERAL RESEARCH OCT., 1998, vol. 13, no. 10, October 1998 (1998-10), pages 1640-1643, XP000891605 ISSN: 0884-0431 the whole document</p>	1-10

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA 99/ 00895

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 3-10 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
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because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

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2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
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Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.



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INTERNATIONAL SEARCH REPORT
Information on patent family members

International Application No

PCT/CA 99/00895

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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WO 9810078 A	12-03-1998	AU 4107397 A	26-03-1998
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19
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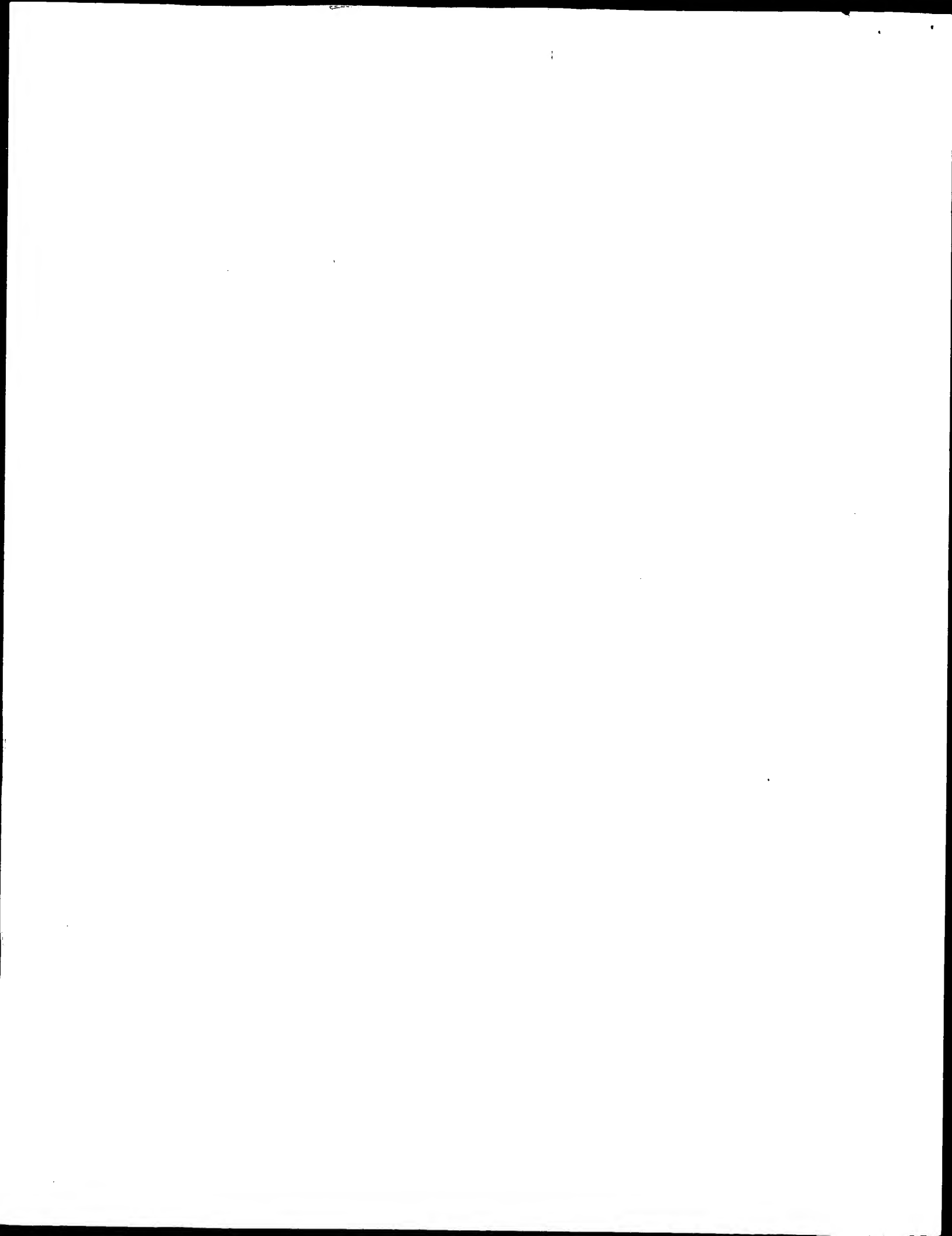
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L5 ANSWER 10 OF 29 EMBASE COPYRIGHT 2002 ELSEVIER SCI. B.V.DUPLICATE 2
ACCESSION NUMBER: 1998194311 EMBASE
TITLE: Cloning of human PEX cDNA: Expression,
subcellular localization, and endopeptidase activity.
AUTHOR: Lipman M.L.; Panda D.; Bennett H.P.J.; Henderson J.E.;
Shane E.; Shen Y.; Goltzman D.; Karaplis A.C.
CORPORATE SOURCE: A.C. Karaplis, Div. of Endocrinology, Sir M.B. Davis-Jewish
Gen. Hospital, McGill University, 3755 Cote Ste-Catherine
Rd., Montreal, Que. H3T 1E2, Canada.
akarapli@ldi.jgh.mcgill.ca
SOURCE: Journal of Biological Chemistry, (29 May 1998) 273/22
(13729-13737).
Refs: 35
ISSN: 0021-9258 CODEN: JBCHA3

COUNTRY: United States
DOCUMENT TYPE: Journal; Article
FILE SEGMENT: 029 Clinical Biochemistry
LANGUAGE: English
SUMMARY LANGUAGE: EnglishL5 ANSWER 11 OF 29 EMBASE COPYRIGHT 2002 ELSEVIER SCI.
B.V.DUPLICATE 3
ACCESSION NUMBER: 1998385394 EMBASE
TITLE: Genetic screening for X-linked hypophosphatemic mice and
ontogenic characterization of the defect in the renal
sodium-phosphate transporter.
AUTHOR: Muller Y.L.; Collins J.F.; Ghishan F.K.
CORPORATE SOURCE: Dr. F.K. Ghishan, Department of Pediatrics, Steele Memorial
Children's Res. Ctr., Univ. of Arizona Hlth. Sci. Center,
1501 N. Campbell Avenue, Tucson, AZ 85724, United States
SOURCE: Pediatric Research, (1998) 44/5 (633-638).
Refs: 18
ISSN: 0031-3998 CODEN: PEREBL

COUNTRY: United States
DOCUMENT TYPE: Journal; Article
FILE SEGMENT: 007 Pediatrics and Pediatric Surgery
022 Human Genetics
029 Clinical Biochemistry
LANGUAGE: English
SUMMARY LANGUAGE: EnglishL5 ANSWER 12 OF 29 EMBASE COPYRIGHT 2002 ELSEVIER SCI.
B.V.DUPLICATE 4
ACCESSION NUMBER: 1998086348 EMBASE
TITLE: Spermine deficiency in Gy mice caused by deletion of the
spermine synthase gene.
AUTHOR: Lorenz B.; Francis F.; Gempel K.; Bsddrich A.; Josten M.;
Schmahl W.; Schmidt J.; Lehrach H.; Meitinger T.; Strom
T.M.
CORPORATE SOURCE: T.M. Strom, Abteilung Medizinische Genetik,
Kinderpoliklinik, Ludwig-Maximilians-Universitat,
Goethestr. 29, 80336 Munchen, Germany.
timstrom@pedgen.med.uni-muenchen.de
SOURCE: Human Molecular Genetics, (1998) 7/3 (541-547).
Refs: 34
ISSN: 0964-6906 CODEN: HMGEE5

COUNTRY: United Kingdom
DOCUMENT TYPE: Journal; Article
FILE SEGMENT: 022 Human Genetics
LANGUAGE: English
SUMMARY LANGUAGE: English5 ANSWER 13 OF 29 BIOSIS COPYRIGHT 2002 BIOSIS
ACCESSION NUMBER: 1998:227575 BIOSIS
DOCUMENT NUMBER: PREV199800227575
TITLE: Pex mRNA is localized in developing mouse



Cloning of Human PEX cDNA

EXPRESSION, SUBCELLULAR LOCALIZATION, AND ENDOPEPTIDASE ACTIVITY*

(Received for publication, February 9, 1998, and in revised form, March 24, 1998)

Mark L. Lipman,^{a,b,c} Dibyendu Panda,^{b,d,e} Hugh P. J. Bennett,^f Janet E. Henderson,^{e,g}
Elizabeth Shane,^h Yingnian Shen,^a David Goltzman,^d and Andrew C. Karaplis^{e,i}*From the Divisions of ^aNephrology and ^bEndocrinology, Department of Medicine, and Lady Davis Institute for Medical Research, McGill University, 3755 Côte Ste Catherine Road, Montréal H3T 1E2, Canada, the ^cCalcium Research Laboratory and ^dEndocrine Laboratories, Royal Victoria Hospital, Department of Medicine, and Sheldon Biotechnology Centre, McGill University, Montréal H3A 1A1, Canada, and the ^eDepartment of Medicine, College of Physicians and Surgeons, Columbia University, New York, New York 10032*

Mutations in the *PEX* gene are responsible for X-linked hypophosphatemic rickets. To gain insight into the role of *PEX* in normal physiology we have cloned the human full-length cDNA and studied its tissue expression, subcellular localization, and peptidase activity. We show that the cDNA encodes a 749-amino acid protein structurally related to a family of neutral endopeptidases that include neprilysin as prototype. By Northern blot analysis, the size of the full-length *PEX* transcript is 6.5 kilobases. *PEX* expression, as determined by semi-quantitative polymerase chain reaction, is high in bone and in tumor tissue associated with the paraneoplastic syndrome of renal phosphate wasting. *PEX* is glycosylated in the presence of canine microsomal membranes and partitions exclusively in the detergent phase from Triton X-114 extractions of transiently transfected COS cells. Immunofluorescence studies in A293 cells expressing *PEX* tagged with a c-myc epitope show a predominant cell-surface location for the protein with its COOH-terminal domain in the extracellular compartment, substantiating the assumption that *PEX*, like other members of the neutral endopeptidase family, is a type II integral membrane glycoprotein. Cell membranes from cultured COS cells transiently expressing *PEX* efficiently degrade exogenously added parathyroid hormone-derived peptides, demonstrating for the first time that recombinant *PEX* can function as an endopeptidase. *PEX* peptidase activity may provide a convenient target for pharmacological intervention in states of altered phosphate homeostasis and in metabolic bone diseases.

X-Linked hypophosphatemic rickets (HYP)¹ is the most com-

mon inherited disorder of renal phosphate wasting characterized by severe hypophosphatemia, renal phosphate wasting, reduced serum concentrations of 1,25-dihydroxyvitamin D levels, and defective bone mineralization (1). Until recently, much of our understanding of HYP has been facilitated by the availability of two murine homologues, the *Hyp* and *Gy* mice, which exhibit many of the phenotypic features of HYP (2, 3). Through positional cloning, however, a gene which spans the deleted region Xp22.1 in HYP patients, or is mutated in non-deletion patients with the disorder, was identified (designated *PEX*) and its partial cDNA sequence reported (4). The predicted human *PEX* gene product, as well as its murine homologue (5), exhibit homology to a family of neutral endopeptidases involved in either activation or degradation of a number of peptide hormones. It has been postulated that *PEX* metabolizes a peptide hormone that modulates renal tubular phosphate handling. Such an activity could involve either the processing of a phosphate-reabsorbing hormone precursor to its active form or the inactivation of a circulating phosphaturic factor. These speculations notwithstanding, the physiologic function of the *PEX* gene product and the mechanisms that lead to the renal and skeletal abnormalities of HYP remain to be defined.

Oncogenous hypophosphatemic osteomalacia (OHO) is a rare acquired disorder of phosphate homeostasis with biochemical and physical abnormalities similar to HYP (reviewed in Ref. 6). This syndrome is associated with a variety of histologically distinct, usually benign, mesenchymal tumors (7) whose excision promptly reverses the metabolic abnormalities and results in cure of the bone disease. It is generally thought that a factor(s) produced by these tumors promotes phosphaturia and inhibits the renal conversion of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D. The nature of the phosphaturic substance remains unknown and is likely distinct from both parathyroid hormone (PTH) and calcitonin, two polypeptide hormones known to inhibit the renal tubular reabsorption of phosphorus (8–13). Because of the striking similarity in the clinical presentation of patients with OHO and HYP, it is postulated that the factor causing phosphaturia in OHO is the active form of the *PEX* substrate. The identification and characterization of the putative *PEX* substrate, referred to as phosphatonin (14), however, will require first a better understanding of *PEX* function. Toward this objective, we have cloned a cDNA encoding

* This work was supported in part by grants from the Medical Research Council of Canada (to M. L. L., J. E. H., D. G., and A. C. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U81970.

^b Contributed equally to the results of this work.

^c Scholar of the Kidney Foundation of Canada.

^d Chercheuse Boursier du Fonds de la Recherche en Santé du Québec.

^e Scholar of the Medical Research Council of Canada. To whom correspondence should be addressed: Div. of Endocrinology, Sir Mortimer B. Davis-Jewish General Hospital, McGill University, Lady Davis Institute for Medical Research, Rm. 626, 3755 Côte Ste-Catherine Rd., Montréal, Québec H3T 1E2, Canada. Tel.: 514-340-8260 (ext. 4907); Fax: 514-340-7573; E-mail: akarapli@lidi.jgh.mcgill.ca.

¹ The abbreviations used are: HYP, X-linked hypophosphatemia;

PEX, phosphate regulating gene with homologies to endopeptidases on the X chromosome; OHO, oncogenous hypophosphatemic osteomalacia; PTH, parathyroid hormone; PCR, polymerase chain reaction; kb, kilobase(s); RT, reverse transcriptase; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography.

the full-length human PEX protein, and determined the tissue distribution of PEX transcripts. In addition, we have examined the subcellular localization of recombinant PEX protein and demonstrated its peptidase activity.

EXPERIMENTAL PROCEDURES

Tumor Tissues Patient I was a 55-year-old woman who presented with a 2-year history of progressively increasing bone pain and difficulty in walking. X-rays of the lumbosacral spine showed diffuse osteopenia. Biochemical investigation showed the serum calcium level to be normal while serum phosphorus was low (0.41 to 0.57 mmol/liter; normal, 0.8–1.6 mmol/liter). Alkaline phosphatase was 232 units/liter (normal, 30–105 units/liter) and tubular reabsorption of phosphate while the patient was hypophosphatemic was decreased to 63% (normal, >80%). A search for a tumor was negative and the patient was treated with 1,25-dihydroxyvitamin D₃ and oral phosphate. Five years later a right hand mass was discovered and was surgically removed. On histopathological examination, it was a fibrous hemangioma. Postoperatively, the patient noted increasing strength in her lower extremities and marked decrease in her pain. The serum phosphorus normalized (0.96 mmol/liter) and the tubular reabsorption of phosphate improved but did not completely normalize (71–76%). No recurrence of the tumor has been found 10 years later.

Patient II was a 21-year-old man with classic features of OHO (15). Resection of a benign extraskeletal chondroma from the plantar surface of the foot resulted in complete reversal of the biochemical and clinical abnormalities associated with the syndrome. Tumor tissue obtained from these two patients at surgery was frozen immediately in liquid nitrogen and stored at -70 °C.

PEX Expression in OHO-associated Tumors—RNA was extracted from tumor tissue using the RNeasy Total RNA kit (Qiagen, Chatsworth, CA) and reverse transcribed using oligo(dT) primer and Superscript II (Life Technologies, Inc.) reverse transcriptase for 1 h at 42 °C in a final reaction volume of 30 µl. The resulting cDNA was then amplified using human PEX-specific oligonucleotide primers PEX-1 (5'-GGAGGAATTGGTTGACGGCG-3') and PEX-2 (5'-GTAGACCACCAAGGATCCAG-3'), designed from the published cDNA sequence (1298 and 1807 are the nucleotide positions of the 5' end of the sense and antisense primers, respectively) (4). Following amplification (35 cycles), an aliquot of the PCR reaction was fractionated on a 1% agarose gel and visualized following staining with ethidium bromide.

Cloning of Full-length PEX cDNA—Cloning of the 5' end of PEX cDNA was accomplished by anchored PCR. Total cellular RNA was extracted from tumor II and mRNA was prepared. 1.5 µg of mRNA was reverse transcribed into cDNA using 100 ng of a PEX-specific antisense oligomer (PEX-2) and 200 units of Superscript II (Life Technologies, Inc.) reverse transcriptase for 1 h at 42 °C in a final reaction volume of 30 µl. The resulting cDNA was size fractionated on a 1% agarose gel and fragments corresponding to >600 base pairs were purified and resuspended in H₂O. The 3' end of the first strand cDNA was homopolymer tailed with dGTP using 1 µl of terminal deoxynucleotidyl transferase at 37 °C for 30 min in a volume of 50 µl. Following heat inactivation of the enzyme, the RNA template was removed by incubation with RNase II and the tailed cDNA was purified by phenol-chloroform extraction followed by ammonium acetate precipitation. The purified tailed cDNA was resuspended in H₂O and an aliquot was used for anchored PCR analysis along with 200 ng of an internal PEX specific antisense primer (PEX-3, 5'-CGTCCCCAGAACTAGGGTCCACC-3'; nucleotide 98 of the published human cDNA sequence is the 5' end of the primer) and 200 ng of oligo(dC) as the sense primer. Forty cycles of PCR were performed using 0.5 µl of Taq polymerase (Promega Biotech, Madison, WI) in a reaction volume of 50 µl. Cycling parameters were: 1 min of denaturation at 94 °C, 2 min of annealing at 55 °C, and 2 min of extension at 72 °C. The PCR products were fractionated on a 1% agarose gel and a band of 700 base pairs was isolated, purified, and ligated into pPCRII vector (Invitrogen). Following transformation into INVαF⁺ bacteria, clones containing the appropriate size insert were sequenced.

To clone the 3' end of PEX cDNA, an aliquot of an amplified unidirectional cDNA library in pCDNA3 vector (Invitrogen) generated from mRNA obtained from tumor I was grown overnight in LB medium and plasmid DNA extracted. DNA (0.5 µg) was subjected to PCR using a PEX-specific sense oligomer (PEX-1) and an antisense oligomer corresponding to the SP6 RNA polymerase-binding site sequences present in the pCDNA3 vector. Thirty-five cycles of amplification were performed in a 50-µl reaction volume with each cycle consisting of 1 min denaturation at 94 °C, 1 min annealing at 55 °C, and 1 min extension at 72 °C. Amplified products were fractionated on a 1% agarose gel and a 1.2-kb

fragment corresponding to the 3' end of PEX cDNA was subcloned and sequenced.

For expression studies, an EcoRV (in the polylinker of pPCRII/AccI (in the PEX sequence) fragment containing the 5' end of PEX cDNA was ligated into the pPCRII vector containing the 3' end of PEX cDNA following digestion with AccI and EcoRV. The resulting plasmid was restricted with KpnI and NotI excising the full-length PEX cDNA that was then inserted into pCDNA3 vector digested at the KpnI/NotI sites in the polylinker region, resulting in plasmid pPEX. The full-length PEX cDNA was sequenced using an Applied Biosystems 373A automated sequencer.

Tissue Expression of PEX mRNA—PEX expression was examined in normal human tissues and in the Saos-2 human osteoblastic osteosarcoma cell line, by RT-PCR using oligonucleotides PEX-4 (5'-CTGGATCCCTGGTGGTCTAC-3') and PEX-5 (5'-CACTGTGCAACTGTCTCAG-3') as sense and antisense primers (2398 and 2895 are the nucleotide positions of the 5' end of these primers designed from the full-length human PEX cDNA). Semiquantitative PCR analysis for PEX expression in human tissues was performed as described previously (16), following normalization for glyceraldehyde 3-phosphate dehydrogenase message in all samples containing PEX transcripts.

Northern Blot Analysis—Total RNA was obtained from tumor I and human Saos-2 osteosarcoma cells using the RNeasy Total RNA kit (Qiagen) and oligo(dT)-purified poly(A)⁺ RNA was isolated from Saos-2 total RNA using standard procedures. Twenty micrograms of tumor I total RNA and 20 µg of Saos-2 poly(A)⁺ RNA were fractionated on 1% denaturing agarose gel, and transferred to nylon membrane (Hybond N⁺, Amersham). Hybridization was performed with ³²P-labeled full-length human PEX cDNA (3.1 kb) in 7 mM Tris-HCl, 50% formamide, 10% dextran sulfate, 4 × SSC, 2 × Denhardt's solution, and heat-denatured salmon sperm DNA (100 µg/ml). The blot was washed in 0.1 × SSC, 0.1% SDS for 20 min at 50 °C, and subjected to autoradiography for 4 days.

In Vitro Transcription, Translation, and Analysis of Products—Plasmid pPEX was linearized with NotI and sense RNA strand was transcribed using T7 RNA polymerase. Translation reactions in rabbit reticulocyte lysate were performed in the presence of [³H]leucine according to the manufacturer's recommendations (Promega) with or without canine pancreas microsomal membranes. Products were analyzed by SDS-polyacrylamide gel electrophoresis (8%). Autoradiography was performed after treating the gel with EN³HANCE (NEN Life Science Products), as described previously (17).

Generation of myc-tagged PEX, Transfection in COS-7 Cells, and Triton X-114 Extraction—Plasmid pPEX-myc was generated by PCR amplification of PEX cDNA using oligonucleotide PexMyc1 as the sense primer (5'-TTGGATGTCAACGCCTCG-3', 519 is the nucleotide position of the 5' end of this primer designed from the cloned human PEX cDNA) and PexMyc2 as the antisense (5'-CTACCACAACTACAGTTGTTTCAGGTCCTCTTCGCTAATCAGCTTTTGTTCATAGAGTCCATGCCCTCTG-3') primer. The latter encodes the human c-myc tag sequences (underlined) and PEX sequences corresponding to the carboxyl-terminal of the mature protein (747RGMDMSMEQKLISEEDLNNCRLW). Following PCR, the amplified fragment was ligated to the pPCR II vector, excised by digestion with KpnI/NotI, and inserted into the corresponding sites in the polylinker region of pCDNA3. The in-frame fusion protein was verified by DNA sequencing.

COS-7 cells maintained in Dulbecco's modified Eagle's medium (DMEM, 4,500 mg/liter glucose with L-glutamine; JRH Biosciences, Lenexa, KS) supplemented with 10% fetal calf serum (Life Technologies, Inc.) and antibiotics (penicillin/streptomycin) were plated at a density of 3 × 10⁵ cells/well in 6-well cluster plates 24 h prior to transfection. Cells were washed with twice with PBS and incubated with 2 µg of pPEX-myc plasmid DNA in 1 ml of DMEM containing 0.1% bovine serum albumin, and DEAE-dextran (Pharmacia) for 3.5 h at 37 °C. Following incubation, the transfection medium was aspirated, the cells were shocked with 10% dimethyl sulfoxide in PBS for 2 min, and then cultured in DMEM with 10% calf serum at 37 °C for 48 h. Triton X-114 extraction were performed on cultured cells expressing myc-tagged PEX as described (18). The samples were then analyzed by immunoblotting using the 9E10 anti-myc monoclonal antibody (19).

Stable Transfection of A293 Cells and Immunofluorescence—A293 cells maintained in DMEM with 10% fetal calf serum were transfected with the pPEX-myc plasmid by electroporation and selection initiated using G418 (600 mg/ml for 14 days and then decreased to 400 mg/ml). Populations of stably transfected cells were recovered at the end of the selection period. For myc-tagged PEX indirect immunofluorescence, stably transfected cells plated on gelatin-coated coverslips were washed twice with PBS, fixed in 4% paraformaldehyde, and in some experi-

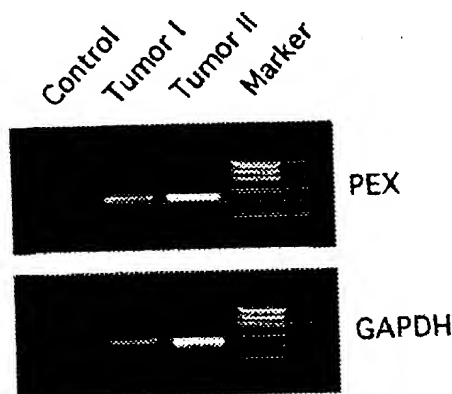


FIG. 1. *PEX* mRNA expression in OHO tumors. Total RNA extracted from two tumors associated with OHO was reverse transcribed and amplified by PCR (35 cycles) using human *PEX*-specific primers, *PEX*-1 and *PEX*-2, designed from the published human sequence. The expected 509-base pair amplified fragment was obtained from both tumor samples. *Control*, no cDNA added to the amplification reaction, i.e. negative control; *Marker*, Φ 174 DNA digested with *Hae*III restriction endonuclease. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

ments permeabilized with 0.5% Triton X-100. Cells were blocked with 10% fetal calf serum in DMEM for 30 min, washed, and incubated for 1 h at 37 °C with the 9E10 anti-myc monoclonal antibody (1:500 dilution). Cells were subsequently washed and incubated in turn with fluorescein-conjugated sheep anti-mouse secondary antibody (1:250 dilution). Coverslips were rinsed extensively with PBS, mounted in medium (glycerol:Tris; 1:1) containing 2.5% 1,4-diazabicyclo-(2,2,2)octane (Sigma) and examined with fluorescent microscopy using appropriate filters.

Assay for Membrane-bound Endopeptidase Activity—COS-7 cells transiently transfected with pCDNA3 vector alone, with vector containing human *NEP* cDNA (generous gift of P. Crine, Université de Montréal), or with pPEX plasmid, were washed and scraped in PBS. Following brief centrifugation, the cell pellets were resuspended in 50 mM Tris-HCl, pH 7.4, and disrupted by sonication. Homogenates were fractionated by sequential centrifugation at $1,000 \times g$ for 10 min and then at $100,000 \times g$ for 60 min. The final precipitate was washed with 50 mM Tris-HCl, pH 7.4, resuspended in the same buffer, and assayed for endopeptidase activity. The protein concentration in membrane fractions was determined by the method of Bradford (20) with bovine serum albumin as standard.

[D-Ala²,Leu⁶]Enkephalin (500 μ M) was incubated with COS cell membrane preparations (~60 μ g of protein) in 100 mM Tris-HCl, pH 7.0, at 37 °C for 30 min (final volume 30 μ l). The reaction was terminated by the addition of 100 μ l of 0.1% trifluoroacetic acid (v/v). Production of Tyr-D-Ala-Gly was monitored using reversed-phase HPLC (Bondapak C-18 reverse phase column, Waters) with a UV detector set at 214 nm. A linear solvent gradient of 0% B to 40% B in 60 min was used with a flow rate of 1.5 ml/min (mobile phase A = 0.1% trifluoroacetic acid (v/v); mobile phase B = 80% acetonitrile, 0.1% trifluoroacetic acid). Tyr-D-Ala-Gly was identified by co-chromatography with marker synthetic peptide. For assessing *PEX* endopeptidase activity, 10 μ g of PTH-(1-38) and PTH-(1-34) peptides (Peninsula Laboratories, Belmont, CA) were added to the membrane preparations. For HPLC analysis of hydrolysis products, a linear solvent gradient of 0% to 50% solution B was used at a rate of 1.5 ml/min. MALDI-TOF mass spectrometry was performed on specific peptide fragments.

RESULTS

Cloning of Human *PEX* cDNA—At the initiation of these studies, *PEX* expression had been reported in minute amounts only in leukocytes and fetal brain. We postulated that in states of hypophosphatemia *PEX* expression may be increased and therefore opted to use the OHO tumor as a tissue source that may express considerably more *PEX*. Tissues obtained from two tumors associated with OHO were used to obtain total RNA and analysis for *PEX* mRNA expression was assessed by RT-PCR. As shown in Fig. 1, *PEX* transcripts were readily

amplified from both tumor samples demonstrating the expected 509-base pair fragment predicted from the published partial human *PEX* sequence (4).

The cloning of the 3' end of *PEX* transcript was performed by rapid amplification of the 3' end of the cDNA, while the 5' of the cDNA was amplified by anchored PCR, as described under "Experimental Procedures." Fig. 2A shows the nucleotide and predicted amino acid sequence of the full-length human *PEX* cDNA cloned from tumor tissues. The composite cDNA reveals a single open reading frame encoding a protein of 749 amino acids which displays homology (34.2% identity, 70% similarity) to human neprilysin (EC 3.4.24.11), and other members of the membrane-bound metalloendopeptidase family encompassing endothelin-converting enzyme-1 (66% similarity) and the Kell antigen (60% similarity), suggesting that *PEX* is a novel member of this family of neutral endopeptidases, as previously suggested (4). Like the other members, *PEX* is a likely a glycoprotein with eight potential N-glycosylation sites and 10 cysteine residues that may be important for the proper folding and hence native conformation of the protein.

The ATG codon at position 604 was assigned as the initiator methionine since it is preceded by two in-frame TGA termination codons 36 and 63 base pairs upstream and conforms favorably to the Kozak consensus for vertebrate initiation of translation (21). The cloned cDNA identifies the first 3 and the last 108 amino acids of the predicted *PEX* gene product in addition to the published partial sequence. These additional amino acids comprise residues such as Glu⁶⁴² and His⁷¹⁰ that are shared by neprilysin, and may be critical for the formation of the active site of the protein and hence its enzymatic activity. Three amino acid residues predicted from our cDNA clone differ from the published partial human *PEX* sequence, D363A (GAC to GCC), R403W (AGG to TGG), and A641G (GCG to GGA). To confirm that these alterations did not arise because of PCR errors, *PEX* sequences were amplified from Saos-2 human osteosarcoma cells (see below) and sequenced. In addition, the same alterations were subsequently described in the murine *Pex* cDNA (12), suggesting possible cloning artifacts in the published partial human *PEX* sequence. Our cloned sequences also encompass 603 nucleotides of the 5'-untranslated region, and 276 nucleotides of the 3'-untranslated region, including the canonical polyadenylation signal AATAAA, 19 nucleotides upstream of the poly(A) tract. The human and the published mouse *PEX* cDNA sequences share extensive homology within the protein coding region (96% identity) as well as in the 5' and 3' noncoding regions.

TMpred analysis of the human *PEX* sequence predicts that the protein has no apparent NH₂-terminal signal sequence but has a single membrane-spanning helical domain comprising amino acid residues 21–39 (Fig. 2C). This predicts its transmembrane topology to be that of a type II integral membrane protein with a 20-residue NH₂-terminal cytoplasmic tail and a COOH-terminal of 700 amino acid residues containing the catalytic domain in the extracellular compartment. Unexpectedly, a CXXX box motif comprising amino acid residues 746CRLW was also identified at the carboxyl terminus of *PEX*. This motif may serve as a site for prenylation, a post-translational lipid modification involved in a number of processes including facilitating membrane attachment, targeting of proteins to specific subcellular membrane compartments, promoting protein-protein interactions, and regulating protein function (22, 23).

Tissue Expression of *PEX* mRNA—We next examined *PEX* expression in a number of fetal and adult tissues and compared the level of expression to OHO tumor RNA using semi-quantitative RT-PCR (Fig. 3). *PEX* transcripts were expressed in

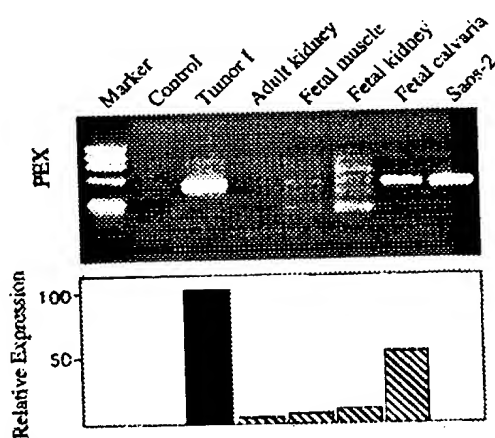


FIG. 3. **PEX expression in human tissues.** Quantitative RT-PCR amplification of the PEX transcripts from total RNA prepared from human tissues and OHIO-associated tumor. Relative expression levels for the PEX transcript were measured by quantifying PEX product in reversed-transcribed RNA samples that have been previously normalized for glyceraldehyde-3-phosphate dehydrogenase levels. The specific primers used were as follows: for PEX, the forward primer was PEX-4 and the reverse primer PEX-5; for glyceraldehyde-3-phosphate dehydrogenase, the primers were as described previously (14). PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. *Control*, negative control; *Marker*, Φ 174 DNA digested with *Hae*III restriction endonuclease. *Below*, shown are the relative levels of PEX transcripts in various human tissues compared with those in the tumor.

human fetal calvarium and to a lesser degree in fetal kidney and skeletal muscle while no expression was apparent in fetal liver (data not shown). PEX expression was also observed in the human osteoblastic osteosarcoma cell line, Saos-2. In adult tissues, PEX mRNA was identified in kidney, but not in liver, or endomyocardium (not shown). Recent studies have also reported PEX expression in human fetal bone, skeletal muscle, and liver as well as fetal and adult ovary and lung (24, 25). Analysis following normalization for glyceraldehyde-3-phosphate dehydrogenase message in all tissues containing PEX transcript disclosed that bone PEX expression is 2–10-fold higher than in other normal tissues examined. In comparison, OHIO tumor PEX expression was twice the levels observed in fetal calvarium, consistent with its relative “overabundance” in these tissues.

Northern Blot Analysis—To determine the size of the full-length PEX transcript, we isolated total RNA from tumor 1 (quantity of available tissue was insufficient for poly(A)⁺ RNA extraction) and poly(A)⁺ RNA from human Saos-2 osteosarcoma cells. This cell line was used since it is readily available and successful amplification of PEX sequences has been performed by RT-PCR (see above). Aliquots (20 μ g of each) were examined by Northern blot analysis using the cloned human PEX cDNA as probe. A single transcript of approximately 6.5 kb was readily detected only in the Saos-2-derived poly(A)⁺ sample and contrasts with the predicted size of the cloned sequence of 3.1 kb (Fig. 4). This finding would therefore predict a ~4 kb 5'-untranslated region for PEX cDNA, consistent with published data from Northern blot analysis of PEX expression in mouse calvaria (5). A less well defined band was also detected in the Saos-2 sample corresponding to a potential tran-

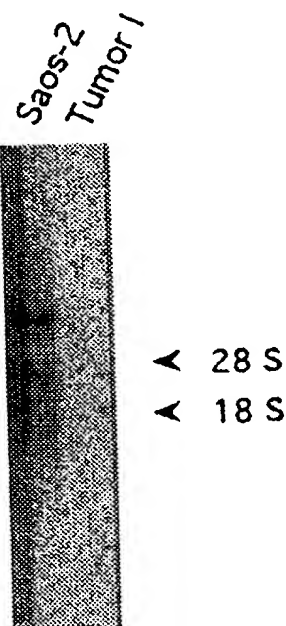


FIG. 4. **Northern blot analysis of PEX mRNA.** Approximately 20 μ g of poly(A)⁺ RNA prepared from Saos-2 cells and 20 μ g of total RNA prepared from tumor 1 tissue were resolved on 1% agarose gel containing formaldehyde and then transferred to a nylon membrane. Following hybridization with radiolabeled PEX cDNA, the blot was washed and the signal detected by autoradiography. A transcript of ~6.5 kb was observed only in the lane containing Saos-2 poly(A)⁺ RNA. There is suggestion of an additional band corresponding to a transcript of ~3.8 kb. *Arrows* indicate the position of the 28 S (approximately 4.8 kb) and 18 S (approximately 1.8 kb) ribosomal RNA.

script of ~3.8 kb, although the nature of this transcript remains unclear. Northern analysis of total RNA samples from tumor 1 and Saos-2 cells (results not shown) did not reveal any signal for PEX, consistent with the relatively low expression levels of the PEX transcript, previously described (4, 24, 25). This finding contrasts sharply with PEX expression levels demonstrated in murine calvaria and cultured osteoblasts (5) and may reflect tissue and species differences.

In Vitro Translation of PEX cRNA—*In vitro* translation studies using full-length human PEX cRNA were performed in the rabbit reticulocyte lysate cell-free system. In the absence of microsomal membranes, PEX cRNA was translated into an ~86-kDa protein, as predicted from the cloned cDNA sequence (Fig. 5). Following addition of canine microsomal membranes to the translation mixture, products of higher molecular mass (~100 kDa) became apparent, consistent with N-glycosylation of PEX at the eight potential glycosylation sites deduced from the predicted sequence.

PEX is a Cell Membrane-associated Protein—Previous studies have established that nephrilysin, endothelin-converting enzyme-1, and Kell blood group glycoprotein are integral membrane proteins. We have used extraction with the detergent Triton X-114 and immunofluorescent localization to examine whether PEX is also a membrane-associated protein. For identification of PEX, we generated a construct in which the carboxyl terminus sequences of PEX are modified by a human

code. The putative start codon is indicated as /1 along with the deduced amino acid translation. Two stop codons preceding the predicted initiation ATG are in **bold type**. **Large asterisk (*)** denotes the putative prenylation site. A potential polyadenylation signal in the 3'-untranslated region is underlined. Eight potential N-glycosylation sites are **boxed**. The sequence has been assigned GenBank accession number U82970. **B**, amino acid homology between PEX and human NEP cDNA. Sequence comparison was performed using the LALIGN program. **C**, TMpred analysis of the PEX sequence showing a single membrane-spanning domain encompassing amino acid residues 21–39 (**arrowhead**). Numbers on the horizontal axis refer to the amino acid sequence.

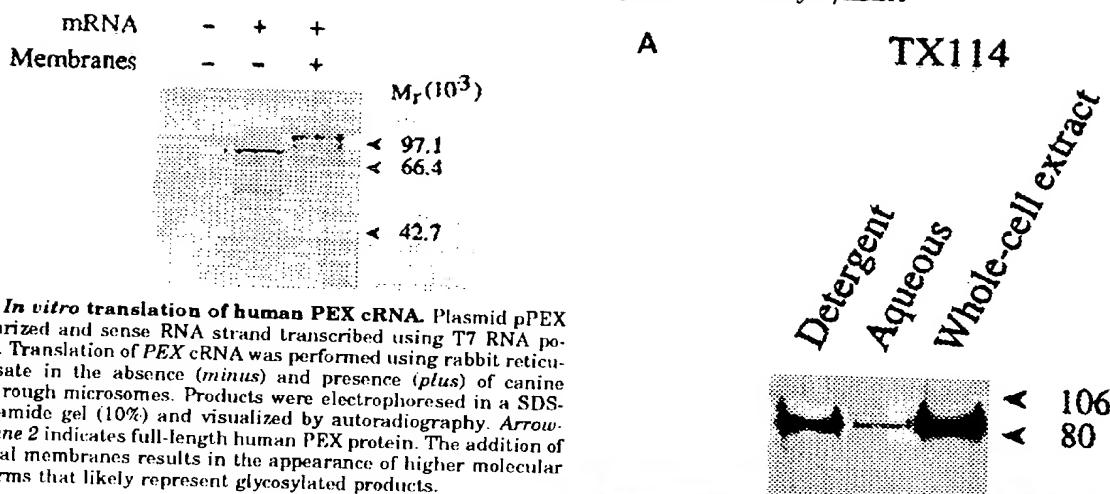


FIG. 5. *In vitro* translation of human PEX cRNA. Plasmid pPEX was linearized and sense RNA strand transcribed using T7 RNA polymerase. Translation of PEX cRNA was performed using rabbit reticulocyte lysate in the absence (*minus*) and presence (*plus*) of canine pancreas rough microsomes. Products were electrophoresed in a SDS-polyacrylamide gel (10%) and visualized by autoradiography. Arrowhead in lane 2 indicates full-length human PEX protein. The addition of microsomal membranes results in the appearance of higher molecular weight forms that likely represent glycosylated products.

c-myc tag. The epitope tag was inserted immediately upstream of the putative prenylation motif so that any potential lipid modification of the PEX protein may proceed uninterrupted.

Triton X-114 is a detergent that forms an aqueous solution at 4 °C but separates into hydrophobic and aqueous phases when the temperature is raised to 30–37 °C. This property has been used as an indicator of the hydrophobic nature of proteins, with integral membrane proteins partitioning exclusively in the detergent phase while highly hydrophilic proteins associate with the aqueous phase. Triton X-114 extracts from COS-7 cells transiently expressing PEX tagged with the *c-myc* epitope showed that PEX partitions nearly exclusively into the detergent phase (Fig. 6A). This finding indicates that PEX is a membrane-associated protein and is consistent with the prediction from sequence analysis that it is an integral membrane protein.

To determine the subcellular localization of PEX, the distribution of recombinant protein expressed in stably transfected A293 cells was examined using immunofluorescence. When cells were fixed and permeabilized, *myc*-tagged PEX immunostaining was detected primarily on the cell surface, but in a number of cells staining was also observed intracellularly, although no signal was observed in the nucleus (Fig. 6B). If permeabilization was omitted, staining was localized exclusively to the plasma membrane (Fig. 6C), while untransfected cells or cells transfected with vector alone showed no immunofluorescent staining (data not shown). Since the *myc*-tag was inserted in the carboxyl end of PEX, these findings further corroborate the sequence-based prediction that PEX is a type II integral membrane protein with its large COOH-terminal hydrophilic domain in the extracellular compartment.

Recombinant PEX Protein Has Endopeptidase Activity—The subcellular localization and sequence similarity between PEX and NEP strongly suggest that PEX functions as a membrane-bound metalloprotease. However, no peptidase activity has been ascribed to PEX. As shown in Fig. 7A, when [D-Ala²,Leu⁵]enkephalin, used to assay for neprilysin activity, was incubated with cell membrane preparations from vector-transfected COS cells or COS cells expressing equivalent amounts of recombinant human neprilysin or PEX proteins, as determined by Western blot analysis (data not shown), production of Tyr-D-Ala-Gly from the substrate was evident only in neprilysin-expressing membrane preparations. While the PEX sequence preserves two of the residues critical for catalytic activity of neprilysin (equivalent to Glu⁶⁴⁶ and His⁷¹¹), it lacks a residue equivalent to Arg¹⁰² shown to be crucial for the dipeptidylcarboxypeptidase activity of neprilysin. Therefore,

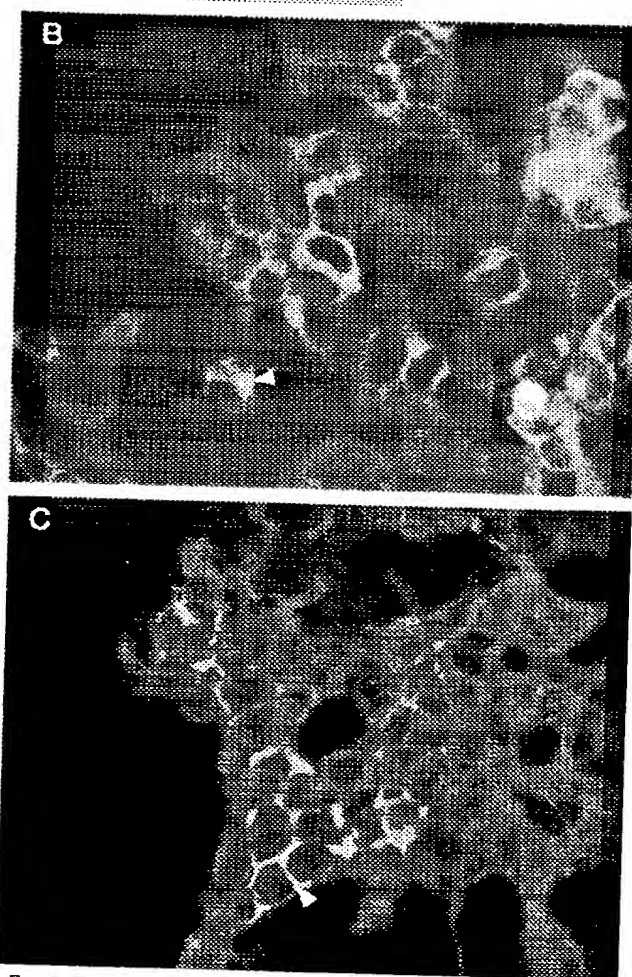


FIG. 6. Triton X-114 extraction and immunofluorescent localization of PEX. A, extraction and partitioning of PEX expressed in COS-7 cells with Triton X-114. Plasmid pPEX-myc was transiently transfected in COS-7 cells and 48 h later cells were extracted with Triton X-114. Whole cell extracts, as well as detergent and aqueous phases, were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotted with an anti-*myc* monoclonal antibody. Right margin indicates molecular mass $\times 10^{-3}$. B and C, localization of PEX using indirect immunofluorescence in stably transfected A293 cells with (B) and without (C) permeabilization with Triton X-100, respectively. Staining was carried out using the 9E10 anti-*myc* monoclonal antibody, followed by fluorescein-labeled secondary (sheep anti-mouse) antibody. Arrowheads indicate intracellular (B) and plasma membrane staining (C).

unlike neprilysin, PEX has no dipeptidylcarboxypeptidase activity, but likely functions as an endopeptidase.

To examine recombinant human PEX for endopeptidase activity, cell membrane preparations from COS cells transiently expressing the protein were incubated with human PTH-(1-38) or PTH-(1-34) and the cleavage products were analyzed by reverse-phase high pressure liquid chromatography (HPLC), as shown in Fig. 8. A parallel preparation from vector transfected COS cells did not appreciably cleave PTH-(1-38). However, in the presence of PEX, both PTH peptides were hydrolyzed in a highly reproducible pattern resulting in the formation of several peaks that absorb at 214 nm. Mass spectrometry of the peptide materials recovered from two product peaks gave m/z values of 861 and 630, respectively. While the former product was present in hydrolysates from both PTH-(1-38) and PTH-(1-34), the latter product was identified only in the PTH-(1-34) hydrolysate and likely corresponds to the carboxyl-terminal pentapeptide DVHNF of human PTH-(1-34). These findings provide the first direct evidence that recombinant PEX possesses endopeptidase activity and suggest that its substrate specificity may not be restricted to the putative phosphatonin but may include other circulating hormones or perhaps bone-derived autocrine/paracrine regulatory factors that regulate renal phosphate handling.

DISCUSSION

To gain insight into the role of PEX in normal physiology we have cloned the human full-length cDNA and studied its expression, subcellular localization, and peptidase activity. The cloned human PEX cDNA encodes a protein whose deduced amino acid sequence is identical to the published partial (4) and to the full-length sequences reported more recently (24-26). Its deduced topology is that of a type II integral membrane glycoprotein and in the present study we have provided experimental evidence to support this prediction. We have shown that PEX is glycosylated in the presence of canine microsomal membranes and partitions exclusively in the detergent phase following extraction with Triton X-114, consistent with the prediction from sequence analysis that it is an integral membrane glycoprotein. Nevertheless, the observed hydrophobic nature of PEX need not be attributed solely to it being an integral membrane protein. Lipophilic modification is known to cause cell membrane association, presumably through hydrophobic interaction of the modifying group with the lipid bilayer. Signaled by the COOH-terminal tetrapeptide CRLW motif, post-translational attachment of isoprenoids via a thioether linkage to the cysteine residue would be sufficient to promote effective membrane association. Further studies will be necessary to determine if such lipid modification of PEX does indeed take place. Of interest, however, is the observation that a nonsense mutation within this motif (R747Stop) has been reported to co-segregate with HYP and is likely to be associated with an inactive PEX gene product (27). Finally, the localization of PEX expressed in A293 cells is also consistent with the protein being membrane-associated and corroborates the sequence-based prediction that PEX is a type II integral membrane protein with its large COOH-terminal hydrophilic domain in the extracellular compartment. While protein expression was detected mostly on the cell surface, in some cells the signal was also localized intracellularly. This localization of the expressed protein would indicate that a portion of PEX activity is located in a membrane-bound compartment, possibly the Golgi membranes. The Golgi localization described for endothelin-converting enzyme-1 activity in cultured endothelial cells (28, 29) is proposed to promote the efficient conversion of big endothelin-1 because of the co-localization and concentration of enzyme and substrate through the constitu-

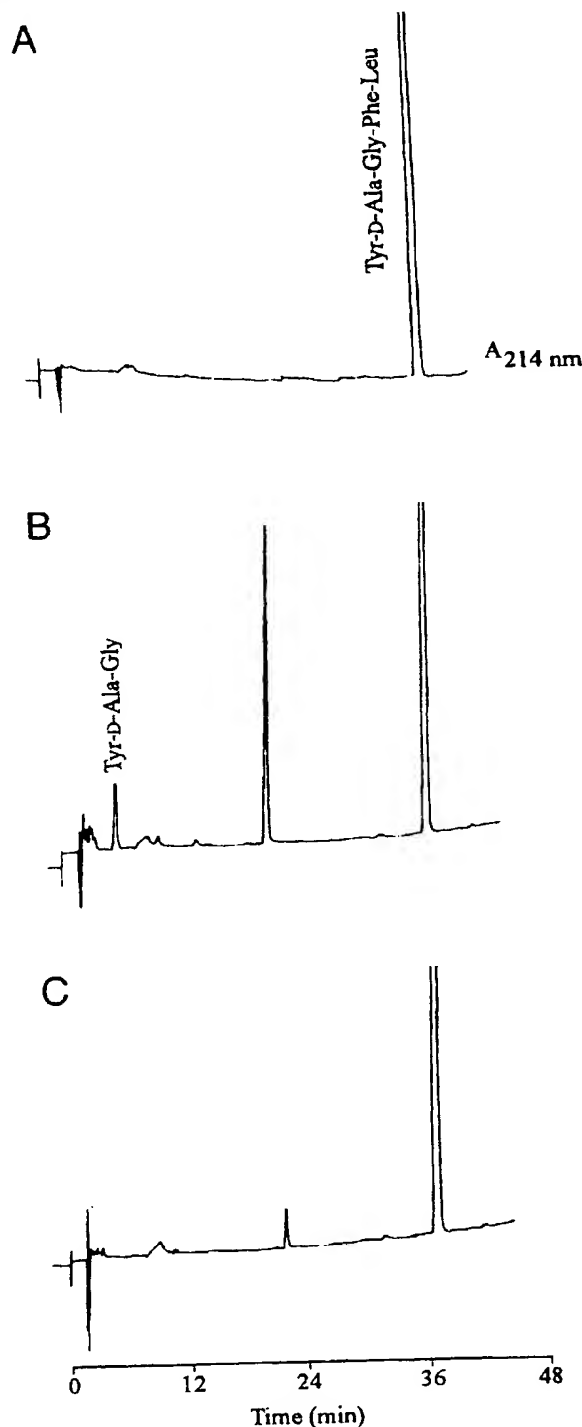


FIG. 7. HPLC analysis of the hydrolysis of [D-Ala²,Leu⁵]enkephalin. A, cell membrane preparations from vector transfected COS-7 cells; or B, from cells transiently expressing human NEP; or C, human PEX cDNAs were incubated in the presence of [D-Ala²,Leu⁵]enkephalin (500 μ M) and hydrolysis products were resolved by HPLC as described under "Experimental Procedures." Tyr-D-Ala-Gly was identified by chromatography of synthetic marker peptide.

tive secretory pathway. It is possible then, that in parallel fashion, the PEX enzyme mediates both intracellular and cell-surface conversions of its putative substrate.

The finding that wild-type PEX transcripts are expressed in

FIG. 8. Hydrolysis of PTH-derived peptides by PEX endopeptidase activity. A, human PTH-(1-38) was incubated with cell membrane preparations from vector transfected COS-7 cells; or B, from cells transiently expressing human PEX and hydrolysis products were resolved by HPLC. C, chromatographic profile of products arising from the hydrolysis of PTH-(1-34) when incubated with cell membranes from COS-7 cells transiently expressing PEX. The novel product with a molecular weight of 630 likely corresponds to the terminal pentapeptide DVHNF of human PTH-(1-34).

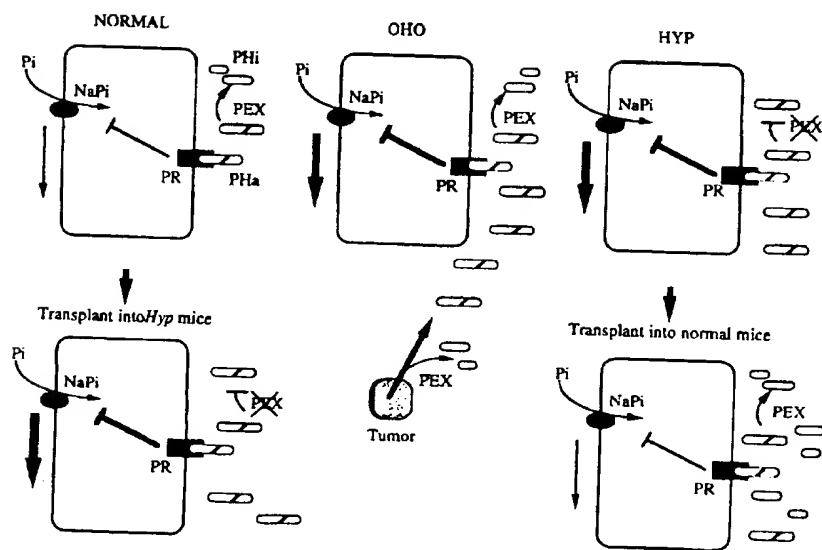
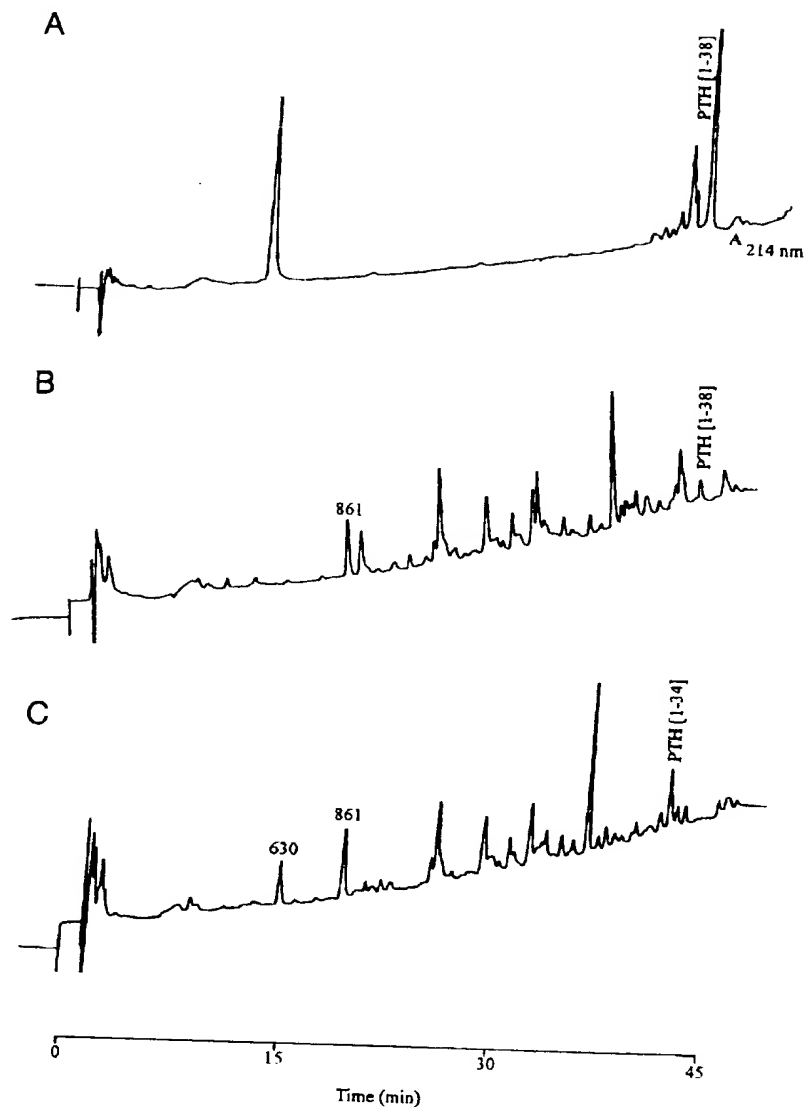


FIG. 9. Schematic representation of phosphate handling in the proximal renal tubule in normal, OHO, and HYP states. The diagrams indicate events proposed to occur at the level of the proximal renal tubule. A putative circulating phosphaturic hormone (PHa) interacts with its renal receptor (PR) and inhibits phosphate reabsorption across the renal brush-border membrane (!) by decreasing NaPi activity. Downward arrows indicate the degree of phosphate excretion. PEX expressed predominantly in extrarenal tissues modulates the levels of circulating PHa by converting it to its inactive form (PHi).

relative overabundance in OHO tumors poses a question in trying to understand the pathophysiology of these disorders. That is, how do we reconcile the apparently disparate observations that overexpression of PEX in OHO and loss of function in HYP patients, both lead to similar derangement in phosphate homeostasis? One of the physiological functions of PEX may well be the inactivation of a factor that normally promotes renal phosphate excretion (Fig. 9). In patients with OHO, the hyperphosphaturia that characterizes the syndrome would be the consequence of unregulated and excessive elaboration of the phosphaturic factor by the tumor. The modestly elevated PEX levels that we have documented in these tumors may arise either in response to the severe hypophosphatemia or to the abnormally high levels of the active phosphaturic factor. Yet, the increased PEX expression may not be sufficient to accommodate the increased substrate load, resulting in abnormally high circulating levels of the active phosphaturic hormone. The inactivation of PEX observed in HYP patients would similarly cause decreased turnover of this humoral phosphaturic factor and thereby lead to renal phosphate wasting.

This model is also consistent with the observation that the *Hyp* phenotype is neither corrected nor transferred following cross-transplantation of kidneys in normal and *Hyp* mice (30). Thus, when *Hyp* mice are engrafted with a normal kidney, phosphaturia ensues since circulating levels of the phosphaturic agent are excessive. On the other hand, engraftment of mutant kidneys in normal mice will not affect renal tubular phosphate handling of the recipients since circulating levels of the phosphaturic substance will be normally regulated by the enzymatic activity of extrarenal wild-type PEX. Indeed, analysis of the tissue distribution of PEX mRNA by RT-PCR has confirmed its expression in extrarenal tissues and particularly bone. Our present findings and those of others (5, 24–26) showing high levels of PEX expression in cells of the osteoblast lineage would be consistent with the intrinsic osteoblast defect postulated to exist in HYP patients (31) and in *Hyp* mice (32, 33).

Finally, although the deduced structure of PEX clearly suggests that it is a metalloprotease, no peptidase activity had been ascribed to the protein. The preservation of the catalytic glutamate and histidine residues (equivalent to Glu⁶⁴⁶ and His⁷¹¹ of neprilysin; Fig. 2B) would argue for such an activity. In addition, the wide range of PEX mutations in HYP patients that align with regions required for protease activity in neprilysin suggests that PEX also functions as a protease (34). Here, for the first time, we provide experimental evidence that recombinant PEX indeed functions as an endopeptidase. Unlike neprilysin, however, the protein does not possess dipeptidyl-carboxypeptidase activity since it lacks a residue equivalent to Arg¹⁰² of neprilysin. Our unexpected observation that PEX effectively degrades PTH raises the question of whether circulating PTH is the putative phosphatonin. Although extracts from some OHO tumors have been reported to stimulate renal adenylate cyclase (15, 35) and this activity was inhibited by PTH antagonists (35), most studies have excluded PTH and PTH-related peptide (PTHrP) activity in OHO-associated tumors. Moreover, calcium homeostasis is generally preserved in patients with HYP. It is more likely, therefore, that the enzyme is rather promiscuous in its substrate specificity. PEX may indeed modulate PTH bioavailability and bioactivity, particularly at the level of the osteoblast, as well as the hormonal and paracrine/autocrine effects of factors produced by osteoblasts

involved in regulating phosphate reabsorption and osteoblast maturation and mineralization. Although additional work will be required to clarify many of these issues, the availability of full-length human PEX cDNA now provides us with the opportunity to study the biology of PEX, identify its substrate(s), elucidate its role in pathological states characterized by dysregulated phosphate homeostasis, and determine its suitability as target for therapeutic intervention in the treatment of metabolic bone diseases.

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AUTHOR(S): Shimozawa, Nobuyuki (1)

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B.V DUPLICATE 6

ACCESSION NUMBER: 1998148625 EMBASE

TITLE: Cellular/molecular control of renal Na/P(i)-cotransport.

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ACCESSION NUMBER: 97099288 EMBASE

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TITLE: Pex/PEX tissue distribution and evidence for a deletion in the 3' region of the Pex gene in X-linked hypophosphatemic mice.

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Review

Oncogenic osteomalacia: is there a new phosphate regulating hormone?

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Summary

Oncogenic osteomalacia is a syndrome associated with rare, usually mesenchymal tumours, which is characterized by hypophosphataemia, phosphaturia and low concentrations of 1,25-dihydroxyvitamin D. The reversal of clinical and biochemical abnormalities following removal of the tumour, indicates it is the source of a humoral factor that is responsible for these abnormalities. It has been demonstrated that the humoral factor inhibits renal phosphate uptake and reduces 1,25-dihydroxyvitamin D production. Although there is evidence that it may act via parathyroid hormone/parathyroid hormone-related peptide receptors and may be a peptide, the factor has not yet been identified, nor has its relationship to factors involved in X-linked hypophosphataemic rickets been established. We propose unifying hypotheses for the pathogenesis of oncogenic osteomalacia and X-linked hypophosphataemic rickets which involve defects in the *PEX* gene. These hypotheses do not fully explain all the available data and it remains possible that hormone(s) with little or no role in X-linked hypophosphataemic rickets may be responsible for oncogenic osteomalacia.

Oncogenic osteomalacia, also known as 'oncogenous osteomalacia' or tumour-induced rickets, is a disorder characterized by the presence of a tumour, hypophosphataemia and low

serum 1,25-dihydroxyvitamin D ($1,25(\text{OH})_2\text{D}$) concentrations with clinical and histological evidence of osteomalacia. Following surgical removal of the tumour, the clinical and biochemical abnormalities disappear (Ryan & Reiss, 1984; Hewison, 1994; Drezner, 1996) indicating that they are due to humoral factor(s) secreted by the tumour.

Oncogenic osteomalacia remains one of the few endocrine syndromes in which the abnormally produced hormone is unknown. Of all the syndromes associated with hypophosphataemia, including hyperparathyroidism, malignancy-associated hypercalcaemia and inherited syndromes of hypophosphataemic rickets, oncogenic osteomalacia most closely resembles X-linked hypophosphataemic rickets (HYP) (Rasmussen & Tenenhouse, 1989). The general consensus has been that the tumours produce a phosphaturic factor responsible for renal phosphate wasting and osteomalacia and that the same or another factor inhibits 25-hydroxyvitamin D (25OHD) $1\text{-}\alpha$ -hydroxylase in the renal proximal tubules (Schapira *et al.*, 1995; Drezner, 1996). However, these factor(s) remain elusive and their relationship (if any) to the material that may circulate in HYP remains unknown.

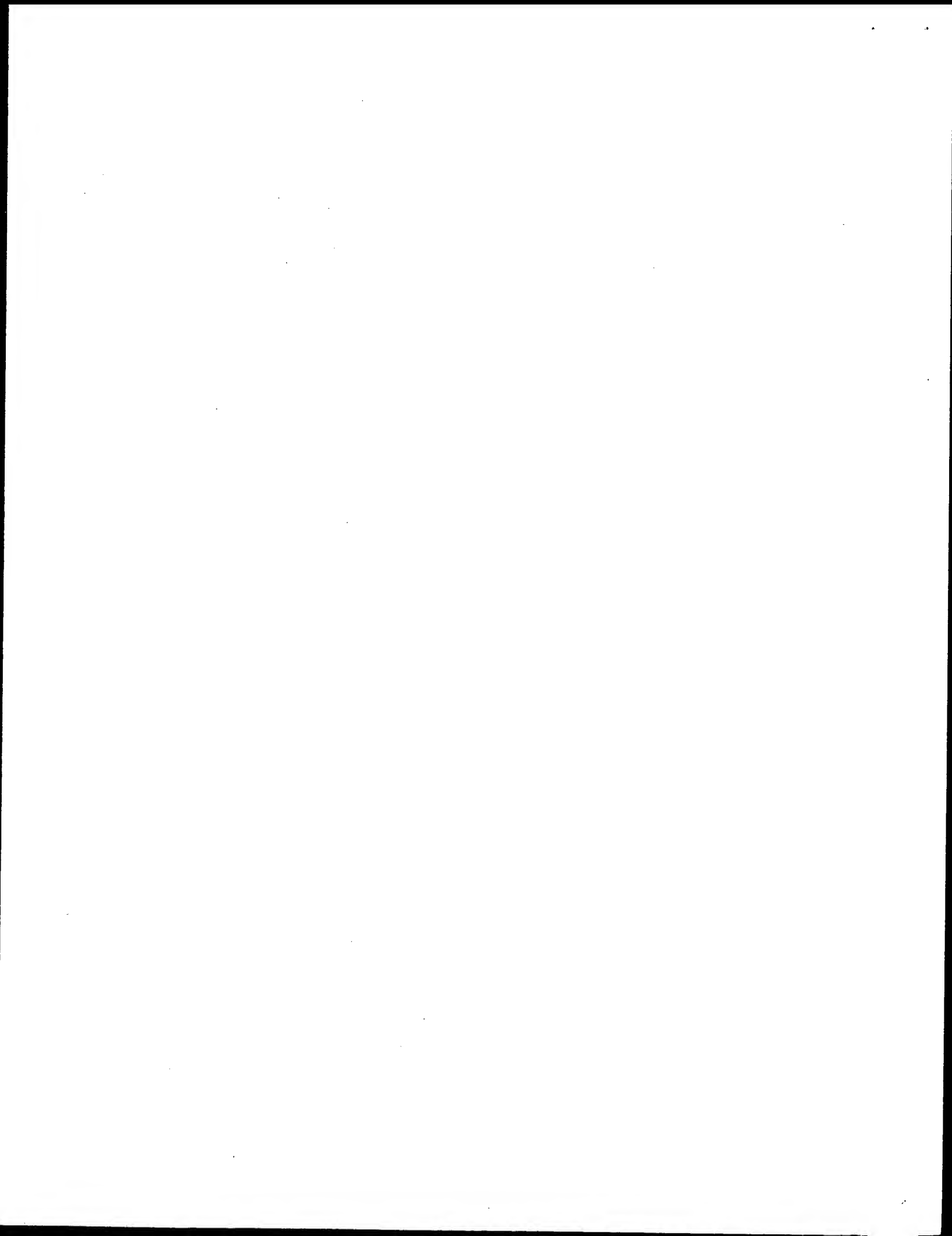
For clinicians, oncogenic osteomalacia remains a diagnostic problem due to difficulties in locating what is commonly a small, obscure, slowly growing tumour and a treatment problem if the tumour cannot be found or completely removed. The other fascination of this apparently rare, but probably under-diagnosed condition, is that identification of the factor responsible may reveal a new hormone.

Clinical presentation

Patients with oncogenic osteomalacia tend to present with vague symptoms that may be of long standing. The clinical presentation can be mistaken for a variety of other conditions including rheumatoid arthritis, muscular dystrophy or a psychiatric disorder (Ryan & Reiss, 1984). The symptoms include generalized pain and muscle weakness which can be severely debilitating (Ryan & Reiss, 1984; Schapira *et al.*, 1995; Drezner, 1996). Other features include recurrent fractures, back pain, waddling gait and difficulty in walking which can ultimately render the patient bedridden (Nitzan *et al.*, 1989).

Typical biochemical findings are hypophosphataemia and phosphaturia due to decreased tubular reabsorption of phosphate. In general, serum concentrations of $1,25(\text{OH})_2\text{D}$ are low or undetectable, although the serum 25OHD concentration is

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normal (Drezner, 1996) and 24,25-dihydroxyvitamin D ($24,25(\text{OH})_2\text{D}$) is also normal (Miyauchi *et al.*, 1988; Cheng *et al.*, 1989). Alkaline phosphatase is commonly high. Glycosuria and aminoaciduria, most commonly glycinuria, are present in some patients (Ryan & Reiss, 1984). Serum calcium and parathyroid hormone (PTH) concentrations are normal in most patients (Weidner, 1991).

How can the tumour be located?

A search for the tumour should start with very thorough physical examination followed by a radiographical survey. CT scan and MRI scan of any clinically suspicious area (Leicht *et al.*, 1993; Lee *et al.*, 1995; Avila *et al.*, 1996; David *et al.*, 1996) and technetium-labelled blood pool scan have also been used successfully to locate the tumour. In some cases, the tumour has been located by the patients themselves (McGuire *et al.*, 1989), so continued follow-up with careful examination is essential. Despite diligent searching, the tumour may not be located (Paterson *et al.*, 1992; Edelson *et al.*, 1993). It is possible that patients diagnosed as sporadic cases of HYP may in fact have oncogenic osteomalacia (Weidner, 1991).

Delays and difficulty in diagnosis are common problems in oncogenic osteomalacia, with delays of up to 19 years reported (Nitzan *et al.*, 1989). The age of onset has been reported from 6 to 88 years (McMurtry *et al.*, 1993; Eyskens *et al.*, 1995), with approximately equal numbers of males and females affected. The tumours are found in a range of sites and can be small and slowly growing. In one case, the tumour was only $1\text{ cm} \times 1\text{ cm}$ and located on the big toe (Moser & Fessel, 1974). One patient was bedridden for 16 years before difficulty in fitting his dentures led to the location of a maxillary tumour (Nitzan *et al.*, 1989).

Heterogeneity of the tumour in oncogenic osteomalacia

The tumours responsible for oncogenic osteomalacia have been described as 'strange tumours in strange places' (Weiss *et al.*, 1985). Although most tumours reported are benign, several malignant cases have been described (Cheng *et al.*, 1989; Harvey *et al.*, 1992; Nakahama *et al.*, 1995) and a case of malignant degeneration of a benign tumour reported (Eyskens *et al.*, 1995). A wide variety of tumours are found both in bone and soft tissue and include mixed mesenchymal tumours, angiosarcomas, chondrosarcomas, prostate carcinoma, schwannoma and neuroendocrine tumours, although most are of mesenchymal origin (Nuovo *et al.*, 1989).

The variation in histology is reflected by the classification of 17 tumours into four morphological groups when examined at a single centre to overcome reporting differences (Weidner &

Santa Cruz, 1987). Most were classified as mixed connective tissue tumours in soft tissue. The other groups were 'osteoblastoma-like', 'nonossifying fibroma-like' and 'ossifying fibroma-like'. Both osteoclast-like giant cells and primitive appearing stromal cells have been observed in the tumours and it has been suggested that the stromal cells are the most likely source of any secreted material (Weidner & Santa Cruz, 1987). Neurosecretory granules, which might have been expected, have not been found in ultrastructural studies of most tumours (Weidner *et al.*, 1985). One report described large cytoplasmic vesicles and it was suggested that the mechanism for hormone secretion may be exocytotic (McClure & Smith, 1987).

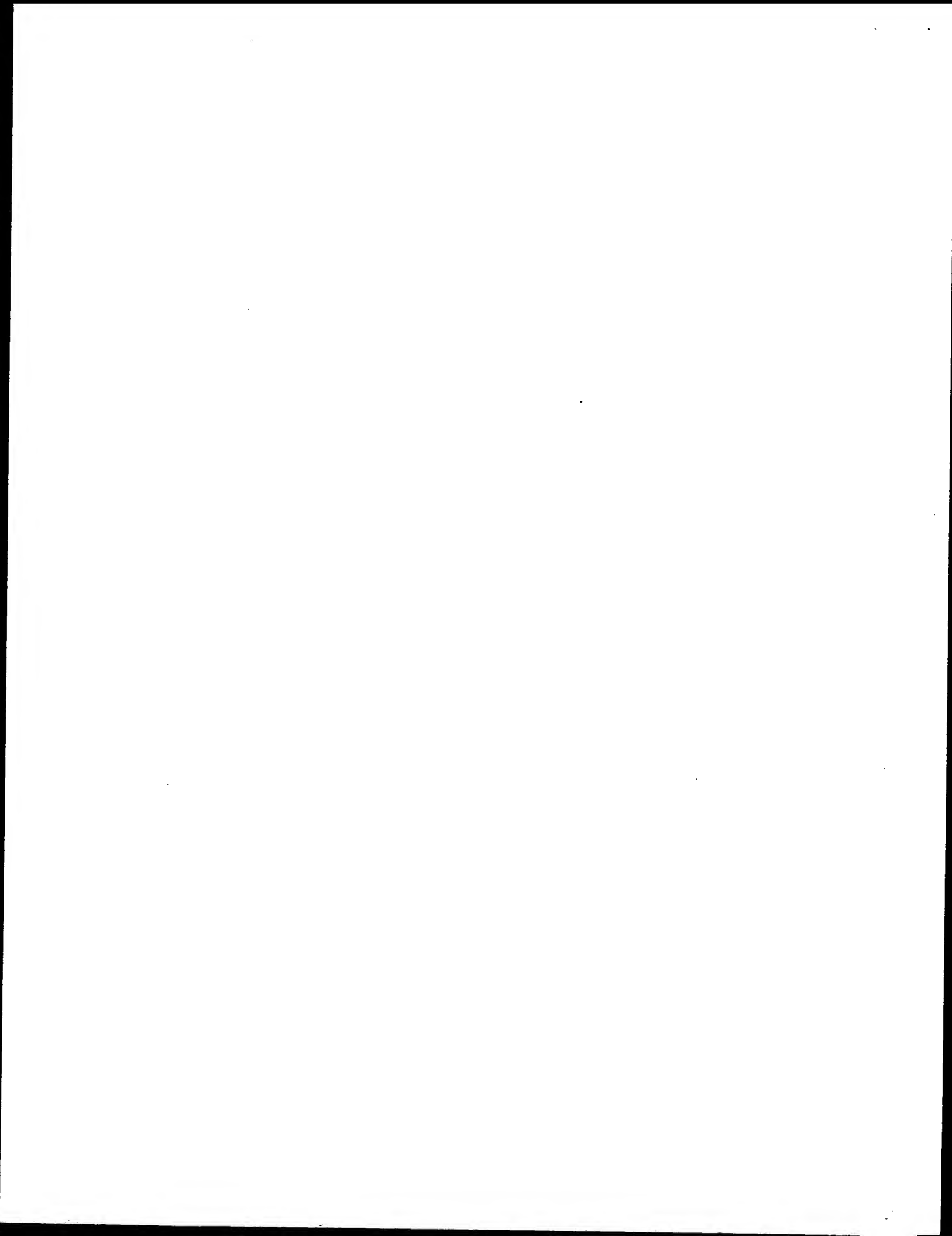
What is known about the mechanism of oncogenic osteomalacia?

Is there a hormone?

The first clue in understanding the pathophysiology of oncogenic osteomalacia comes from the observation that tumour removal produces reversal both of symptoms and the abnormal biochemical profile. Furthermore, if the tumour cannot be located or completely removed, the most effective treatment is to administer both phosphate and $1,25(\text{OH})_2\text{D}$ (Drezner, 1996). This is consistent with the proposal that the tumour produces a factor, or factors, responsible for both renal phosphate wasting and the low concentration of $1,25(\text{OH})_2\text{D}$.

Does the hormone cause phosphaturia in vivo and in vitro?

Early experiments *in vivo* demonstrated phosphaturia following injection of tumour extracts into a dog (Aschinger *et al.*, 1977), into parathyroidectomised mice (Lau *et al.*, 1979) and into both intact and parathyroidectomised rats (Popovtzer, 1981). Heterotransplantation of tumour tissue into athymic nude mice has been shown to result in hypophosphataemia and phosphaturia by several groups (Drezner *et al.*, 1982; Miyauchi *et al.*, 1988; Nitzan *et al.*, 1989). More recent studies have shown inhibition of phosphate uptake in opossum kidney (OK) cells by conditioned media collected from cultured oncogenic osteomalacia tumour cells (Cai *et al.*, 1994; Wilkins *et al.*, 1995; Rowe *et al.*, 1996). Our laboratory has also shown inhibition of phosphate uptake by conditioned media in the highly PTH-sensitive OK3B2 cell line (Nelson *et al.*, 1996a). These studies indicate that a factor secreted by the cells directly inhibits phosphate reabsorption by renal cells and the name 'phosphatonin' has been suggested for this phosphate uptake inhibitory factor (Econs & Drezner, 1994).



Does the hormone affect 1-hydroxylation *in vivo* and *in vitro*?

Reduced activity of 25OHD 1- α -hydroxylase has been shown *in vivo* in tumour transplanted mice (Drezner *et al.*, 1982) and *in vitro* in primary cultures of renal cells treated with tumour extract (Miyauchi *et al.*, 1988). To date, no reduction in 25OHD 1- α -hydroxylase activity has been reported in response to tumour cell conditioned medium (Rowe *et al.*, 1996).

Is the hormone a peptide?

Miyauchi *et al.* (1988) showed that the activity of tumour extract was heat sensitive and lipid insoluble. Activity of conditioned medium was also shown to be heat sensitive (Wilkins *et al.*, 1995; Nelson *et al.*, 1996a). In our studies activity was sensitive to trypsin at 50 μ g/ml for 2 h; in other studies where activity was not trypsin sensitive, lower concentrations (Miyauchi *et al.*, 1988) or shorter incubation times (Wilkins *et al.*, 1995) were used. Dialysis of conditioned medium has indicated a molecular weight between 8 and 25 kD (Cai *et al.*, 1994). Taken together these results suggest that the factor may be a peptide hormone.

Is the hormone similar to known regulators of phosphate transport?

Immunoassay of conditioned media by Wilkins *et al.* and in our laboratory did not detect parathyroid hormone-related peptide (PTHrP) or PTH (Wilkins *et al.*, 1995; Nelson *et al.*, 1996a). Cai *et al.* reported PTH-like immunoreactivity in conditioned media, although serial dilutions showed it was neither authentic PTH nor an N-terminal fragment (Cai *et al.*, 1994). In our laboratory, Northern blot analysis of RNA prepared from cultured tumour cells showed no hybridization to PTH or PTHrP riboprobes, nor to a probe for the recently described putative phosphate regulator, human stanniocalcin (Nelson *et al.*, 1996a).

How does the hormone act?

Stimulation of cAMP in response to tumour extracts from three patients with oncogenic osteomalacia has been demonstrated in a PTH-sensitive chick renal membrane adenylate cyclase system (Seshadri *et al.*, 1985). Except for this study there is no evidence that cAMP is involved in the renal effects of the active factor. Neither our studies nor those of Cai *et al.* showed cAMP stimulation in OK cells in response to tumour cell conditioned media that inhibited phosphate uptake, or suppression of phosphate uptake inhibitory activity by the PTH analogue 8,18 Nle, 34 Tyr bovine 3-34 PTH (3-34 bPTH) (Cai *et al.*, 1994; Nelson *et al.*, 1996a). These latter results may not

exclude involvement of PTH/PTHrP receptors in the activity of the factor, because it has also proved difficult to suppress PTH and PTHrP-induced inhibition of phosphate uptake in OK cells using 3-34 bPTH as antagonist (Muff *et al.*, 1990). Evidence for the involvement of PTH/PTHrP receptors comes from the demonstration by Seshadri *et al.* (1985) that the renal membrane adenylate cyclase activity stimulated by tumour extracts, was suppressed by 3-34 bPTH. Our studies also showed that 3-34 bPTH suppressed the cAMP stimulation by conditioned media in an osteosarcoma cell line (Nelson *et al.*, 1996a). The inability of Nitzan *et al.* (1989) to detect phosphate uptake inhibitory activity using the JTC-12 cell line, that is not sensitive to PTH (Malmstrom & Murer, 1986), and our demonstration of marked activity in a highly PTH-sensitive renal cell line (Nelson *et al.*, 1996a) are also consistent with the hypothesis that PTH/PTHrP receptors may be involved in mediating the activity of the factor.

How close is the identification of the hormone?

Two groups have reported preliminary isolation of putative factors. Kumar *et al.* (1995) have isolated a cDNA clone, HEM-1, from a cDNA library prepared from cultured oncogenic osteomalacia tumour cells on the basis of PTH-like immunoreactivity. Transfection of OK cells with HEM-1 did not affect phosphate uptake and no expression of HEM-1 RNA was demonstrated in tumours or tumour cell cultures. A rabbit reticulocyte lysate was used to translate protein from HEM-1 synthetic mRNA, however, no functional studies of the product were described. We have designed PCR primers based on the sequence of HEM-1 and amplified a product of the predicted length and sequence from a cDNA library prepared from our cultured tumour cells. We have not, however, been able to detect hybridization of the PCR product to tumour cell RNA on Northern blot (Nelson *et al.*, 1996b). Recently Rowe *et al.* (1996) reported the isolation of two proteins of 56 and 58 kD on Western blot in conditioned media from day 1 cultures of haemangiopericytoma cells when screened with antisera raised from preoperative but not postoperative whole serum collected from the patient before and after surgical tumour removal. No further characterization or functional studies of these proteins has yet been reported.

Is there a relationship to HYP?

Similarities between HYP and oncogenic osteomalacia

The clinical presentation of oncogenic osteomalacia is very similar to that of the X-linked dominant inherited condition HYP (Rasmussen & Tenenhouse, 1989). Patients with HYP

also exhibit hypophosphataemia associated with renal phosphate wasting, resulting in deficient bone mineralization and rickets. The concentration of $1,25(\text{OH})_2\text{D}$ in HYP is inappropriately normal, in view of hypophosphataemia (Mason *et al.*, 1982).

Evidence for a humoral factor in Hyp mice

Many studies into the pathophysiology of HYP have been carried out using the murine models of X-linked hypophosphataemia, Hyp and Gy mice. Experiments with Hyp mice have provided evidence for a humoral factor. Parabiosis experiments demonstrated that the defect could be passed from Hyp mice to normal mice (Meyer *et al.* 1989a) and that this was unaffected by parathyroidectomy (Meyer *et al.* 1989b). Results of cross transplantation experiments of kidneys between normal and Hyp mice were also consistent with the hypothesis of a humoral factor (Nesbitt *et al.*, 1992). While sodium-dependent phosphate uptake is reduced in renal brush border membrane vesicles from Hyp mice compared with controls (reviewed, Tenenhouse & Beck, 1996), studies of immortalized cells not exposed to possible hormonal influences, suggest there is not an intrinsic defect in renal phosphate transport in Hyp mice (Nesbitt *et al.*, 1995; Nesbitt *et al.*, 1996). The lack of a gene dose effect in Hyp mice (Qiu *et al.*, 1993) is also consistent with a humoral factor. In HYP patients there is evidence for a gene dose effect in mineralization of bone and teeth, although there are no differences between males and females in renal phosphate handling (Scriver & Tenenhouse, 1992). The recent demonstration of inhibition by Hyp mouse serum of phosphate uptake by primary mouse proximal tubular cells also provides evidence for a circulating phosphaturic factor in Hyp, a factor which may be produced in part by osteoblasts (Lajeunesse *et al.*, 1996).

Despite the considerable evidence for a humoral factor in Hyp mice, there is also evidence for intrinsic osteoblast abnormalities (Hruska *et al.*, 1995). Transplanted osteoblasts from Hyp mice produce abnormal bone with impaired mineralization. Phosphate deprivation and supplementation studies have shown that hypophosphataemia is not the only cause of the abnormal bone mineralization and provide evidence for an osteoblast dysfunction in the Hyp mouse (Ecarot *et al.*, 1992).

Differences between HYP and oncogenic osteomalacia

Based on the similarities of clinical presentation and evidence for humoral involvement in both conditions, it has been proposed that the same factor may be responsible for both oncogenic osteomalacia and HYP (Econs & Drezner, 1994;

Hewison, 1994; Rowe, 1994). There are some differences, however, between the two conditions. In clinical presentation, dental abnormalities, particularly tooth abscesses, are common in patients with HYP but have not been described in oncogenic osteomalacia. In HYP the level of $1,25(\text{OH})_2\text{D}$ is normal, but not low or undetectable, as described in many cases of oncogenic osteomalacia. The abnormalities of $1,25(\text{OH})_2\text{D}$ metabolism appear to differ in the two conditions. In oncogenic osteomalacia there is evidence for inhibition of 25OHD 1- α -hydroxylase (Drezner *et al.*, 1982; Miyauchi *et al.*, 1988) whereas in Hyp mice elevated catabolism of $1,25(\text{OH})_2\text{D}$ due to increased activity of renal 25(OH) D -24-hydroxylase (24-hydroxylase) has been shown. In Hyp mice, catabolism is further elevated following phosphate deprivation resulting in reduced concentrations of $1,25(\text{OH})_2\text{D}$ (Tenenhouse & Jones, 1990).

Involvement of renal sodium-dependent phosphate (Na/Pi) type II cotransporters may also differ between HYP and oncogenic osteomalacia. These transporters, which include NaPi2 (rat) and NaPi4 (OK cell), are largely responsible for proximal tubular phosphate reabsorption and its regulation by PTH and by phosphate deprivation (Murer & Biber, 1996). Reduced levels of NaPi2 mRNA and protein have been demonstrated in the proximal tubules of Hyp mice compared with controls (Collins & Ghishan, 1994; Tenenhouse *et al.*, 1994). These findings were confirmed in Gy mice by one group (Beck *et al.*, 1996) although another group could not demonstrate reduced NaPi2 mRNA levels (Collins & Ghishan, 1996). In our laboratory we have detected no changes in expression of NaPi4 mRNA in OK cells in response to oncogenic osteomalacia tumour conditioned media with phosphate uptake inhibitory activity (unpublished data).

Implications of the identification of PEX as the candidate gene for HYP

The hypothesis that a single common humoral factor may explain both HYP and oncogenic osteomalacia has now been challenged by the identification of *PEX* as the candidate gene for human HYP (Francis *et al.*, 1995). The *PEX* gene has strong homology to a family of neutral, membrane-bound endopeptidases. Mutations identified in patients from HYP families include large deletions, suggesting that the gene is not functional in affected individuals. The mouse *Pex* gene has also been isolated (Du *et al.*, 1996) and deletions in the 3' region identified in the Hyp mouse and in the 5' region in the Gy mouse (Strom *et al.*, 1997). In many families with HYP, no mutation in the coding sequence has been found to date (Holm *et al.*, 1997; Rowe *et al.*, 1997). Mutations may occur in upstream sequence, which is also deleted in the Gy mice, which may affect regulation of *PEX*. A number of possible pathways

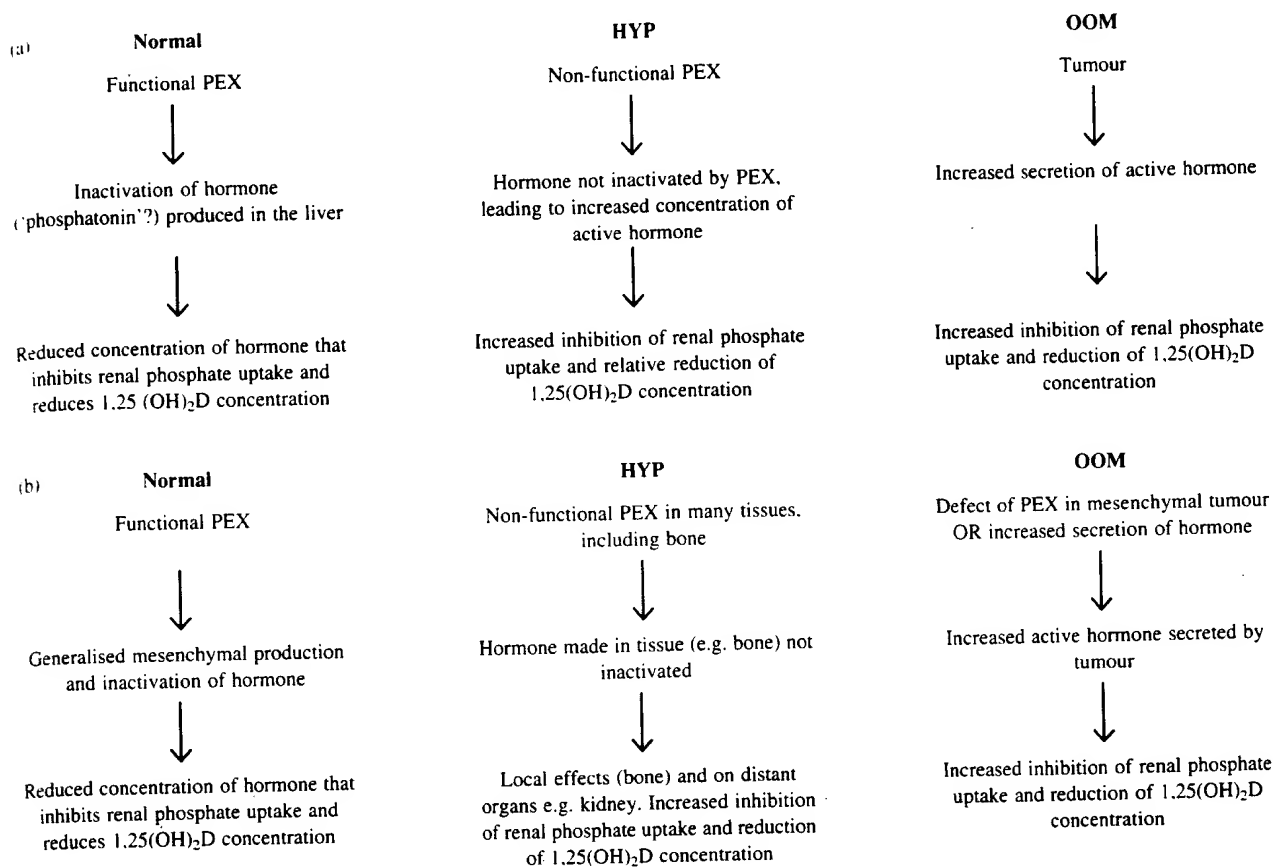


Fig. 1 Hypotheses for the mechanism of oncogenic osteomalacia (OOM) and X-linked hypophosphataemic rickets (HYP). (a) proposes inactivation by PEX endopeptidase of a hormone, possibly produced in the liver. (b) proposes local and hormonal effects of a hormone produced in many mesenchymal tissues and inactivated by PEX endopeptidase.

involving PEX in the pathogenesis of HYP have been proposed (Econs, 1996; Nesbitt & Drezner, 1996).

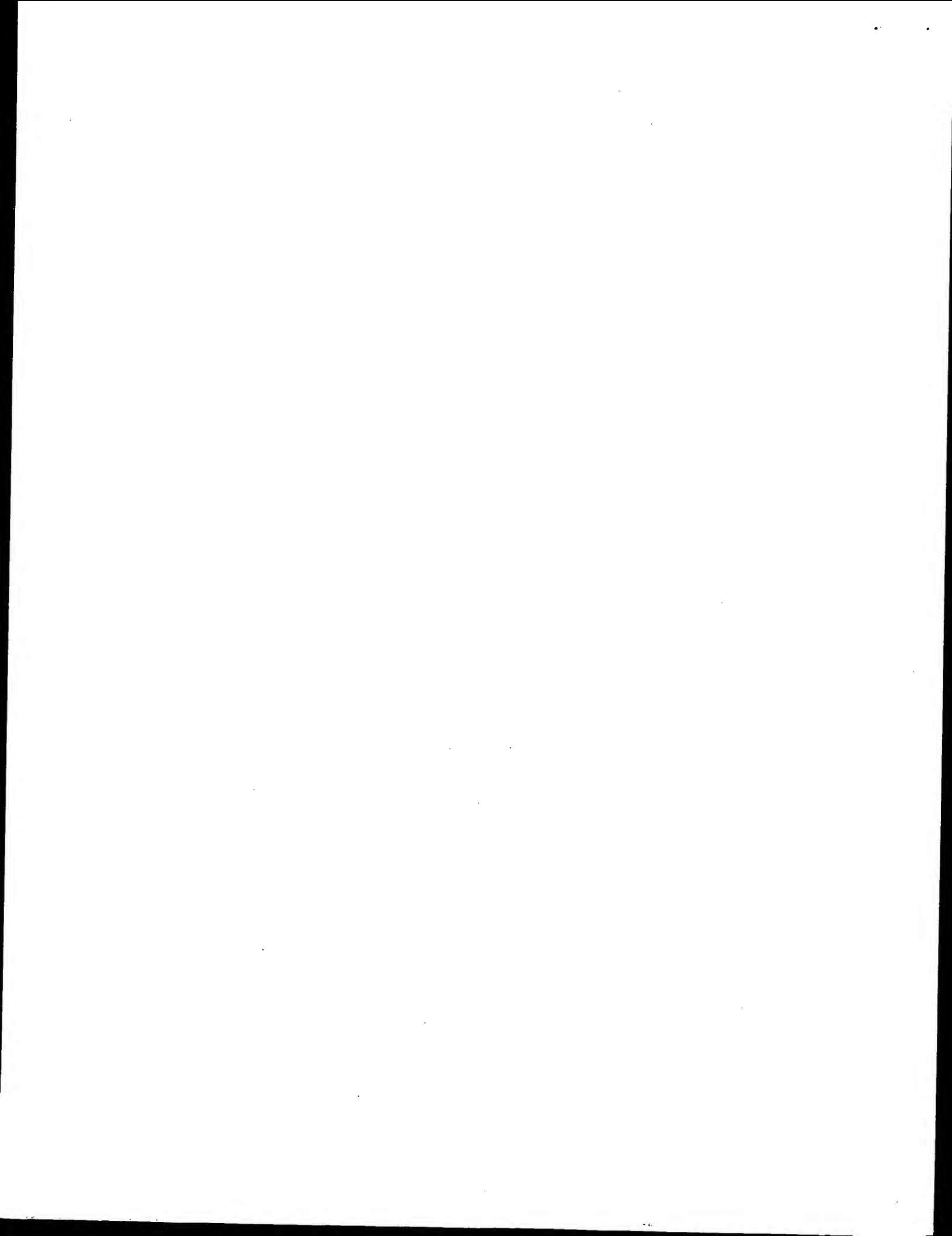
Hypotheses for the mechanism of HYP and oncogenic osteomalacia

Unifying hypotheses for how the PEX gene may explain HYP and oncogenic osteomalacia

Based on the homology of PEX with the neutral endopeptidases, it is likely that the PEX gene product activates or inactivates another protein. One hypothesis (Fig. 1a) is that membrane-bound PEX endopeptidase normally inactivates a hormone, possibly produced in the liver, that inhibits renal phosphate uptake and regulates renal 1,25(OH)₂D metabolism, either by inhibition of synthesis by 25OHD 1- α -hydroxylase or by increased catabolism by 24-hydroxylase. Lack of functional PEX endopeptidase in HYP would lead to an increased plasma concentration of the hormone and therefore to increased

inhibition of renal phosphate uptake and to lower than expected 1,25(OH)₂D concentrations. In oncogenic osteomalacia there may be inappropriately high secretion of the hormone by the mesenchymal tumour, also leading to renal phosphate wasting and reduced level of 1,25(OH)₂D. Evidence in favour of this hypothesis is provided by the recent report of production of a phosphate uptake inhibitory factor ('phosphatonin') by hepatocytes of Hyp mice (Nesbitt & Drezner, 1996).

A related hypothesis (Fig. 1b) can be proposed in which there is normally constitutive production of the hormone which is inactivated by PEX in a number of mesenchymal tissues, including bone and teeth. Lack of functional PEX, leading to an increased level of the hormone in bone, could lead to local effects on osteoblast metabolism and bone formation. This is consistent with the intrinsic defects observed in osteoblasts in Hyp and with the demonstration that conditioned medium from Hyp mouse primary osteoblast-like cultures inhibited phosphate uptake by mouse proximal tubular cells (Lajeunesse *et al.*, 1996). Expression of PEX/Pex



mRNA has been reported in a range of human foetal and adult mouse tissues (Beck *et al.*, 1997). The expression appeared to be at low levels and to be predominantly in bone. These studies did not detect expression in kidney or liver. In oncogenic osteomalacia, either a somatic defect in the *PEX* gene in the mesenchymal tumour, or increased production of the hormone by the tumour, could result in an increased level of the active hormone.

A second hypothesis is that *PEX* activates a stimulator of renal phosphate uptake. In HYP, lack of functional *PEX* would lead to a reduced level of active hormone and therefore to loss of phosphate by the kidney. It is possible that human stannocalcin, which stimulates renal phosphate uptake in rats (Olsen *et al.*, 1996), may also have the same effect in humans. We were unable, however, to detect hybridization of oncogenic osteomalacia tumour cell RNA to a probe for human stannocalcin on Northern blot analysis (Nelson *et al.*, 1996a). Furthermore, this hypothesis does not explain the presence of a factor that inhibits renal phosphate uptake in conditioned media from oncogenic osteomalacia tumours.

Other possibilities for the mechanism of HYP and of oncogenic osteomalacia

It seems likely that even if one of these hypotheses is the basis of the mechanism for HYP and oncogenic osteomalacia the mechanism may be more complex, as neither clearly explains all the observed data.

The hypotheses of activation or inactivation of a phosphate regulatory hormone by membrane-bound *PEX* endopeptidase do not easily explain the results of parabiosis experiments with Hyp mice. It might be expected that functional *PEX* in the normal mouse would inactivate the excess phosphate wasting hormone, or activate the phosphate conserving hormone from the Hyp mouse. Parabiosis of Hyp to normal mice did not normalize the Hyp phenotype, however, and the defect was also transferred to the normal mice. It is possible that the increased load of the substrate from the affected mouse may have exceeded the threshold of the normal mouse endopeptidase or that the circulating hormone acted on end organs such as the kidney and bone before inactivation at another site in the normal mouse.

The hypotheses also do not explain the differences previously described between HYP and oncogenic osteomalacia in clinical presentation and abnormalities of 1,25(OH)₂D metabolism. The relationship between the renal phosphate wasting and the abnormalities in vitamin D metabolism in both conditions is also unknown. These may both result from separate direct effects of a single hormone, via a common signalling mechanism, or via two different hormones.

Conclusion

At this stage therefore it is not clear how mutations in the *PEX* gene explain X-linked hypophosphataemic rickets in humans. Although there are many phenotypic similarities between oncogenic osteomalacia and HYP, it is also not clear how HYP and mutations in the *PEX* gene relate to oncogenic osteomalacia. What cannot be excluded is that a hormone with no role in HYP may be secreted in oncogenic osteomalacia and result in renal phosphate wasting and a low level of 1,25(OH)₂D. Studies from tumour cell lines that produce a phosphate uptake inhibitory factor may provide crucial insights into this interesting syndrome and possibly reveal a new phosphate regulating hormone.

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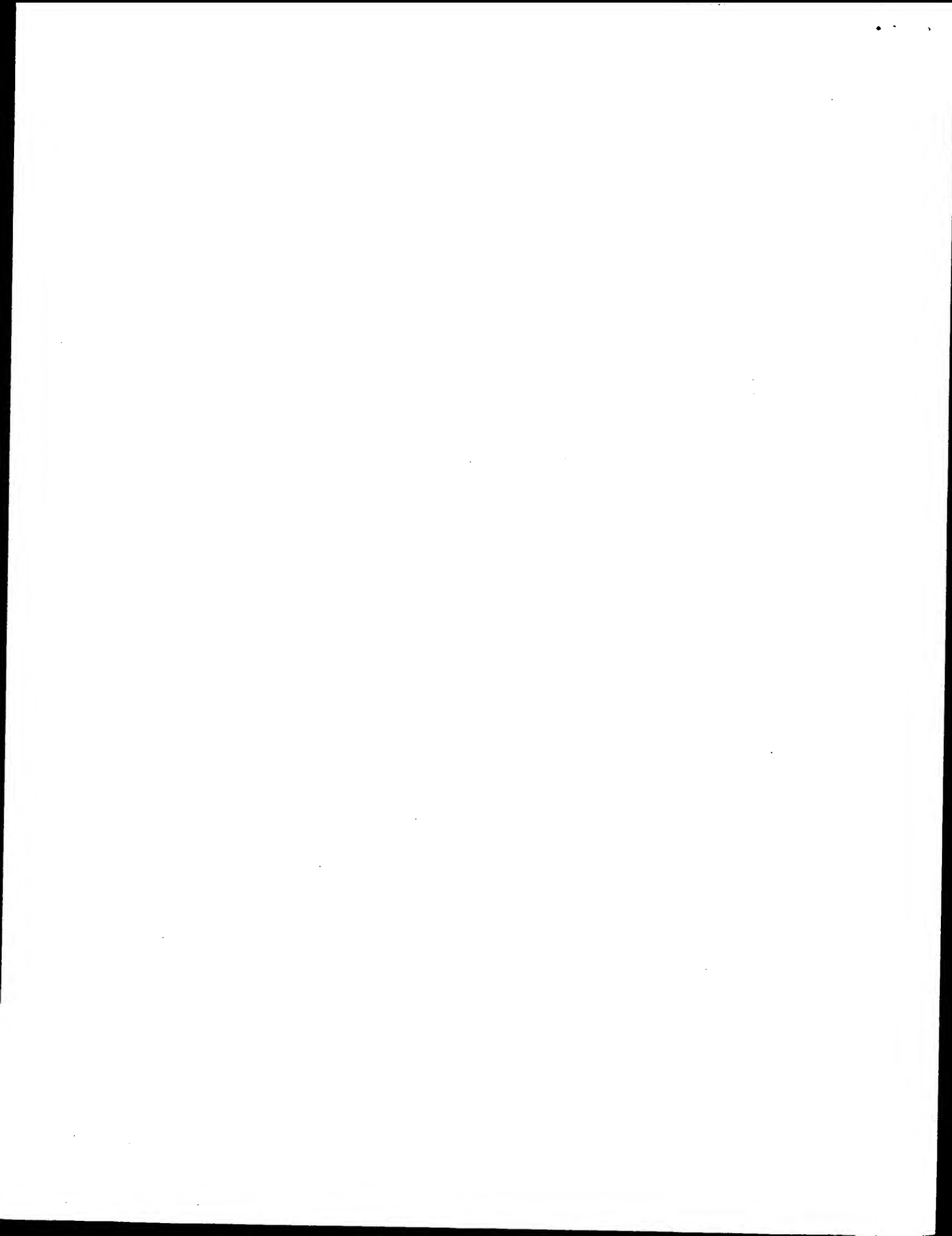
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TITLE: Pex/PEX tissue distribution and evidence for a deletion in the 3' region of the Pex gene in X-linked hypophosphatemic mice.

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AUTHOR: Nelson A.E.; Robinson B.G.; Mason R.S.

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ARTICLE

Pex mRNA Is Localized in Developing Mouse Osteoblasts and Odontoblasts

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SUMMARY Mutations in PEX, a phosphate-regulating gene with homology to endopeptidase on the X chromosome, were recently identified in patients with X-linked hypophosphatemia (XLH), an inherited disorder of phosphate homeostasis characterized by growth retardation and rachitic and osteomalacic bone disease. To understand the mechanism by which loss of PEX function elicits the mutant phenotype, a study of its mRNA localization and ontogenesis was undertaken. Using the reverse transcriptase-nested polymerase chain reaction (RT-nested PCR) with polyA⁺ RNA purified from mouse testis, a 337-bp Pex cDNA fragment was generated and cloned in the pCRII plasmid. The cDNA was used to generate sense and anti-sense Pex riboprobes for in situ hybridization (ISH) and Northern analysis. To survey a large number of different tissues, sagittal sections of embryos and newborn mice were examined. ISH showed the presence of Pex mRNA in osteoblasts and odontoblasts. Pex gene expression was detectable on Day 15 of embryonic development, which coincides with the beginning of intercellular matrix deposition in bones. Finally, Northern analysis of total RNA from calvariae and teeth of 3-day-old and adult mice showed that the abundance of the 7-kb Pex transcript is decreased in adult bones and in nongrowing teeth. The present study demonstrates that Pex mRNA is expressed in bones and teeth and suggests that this putative endopeptidase plays an important role in the development of these tissues. (*J Histochem Cytochem* 46:459-468, 1998)

KEY WORDS

bone
teeth
extracellular matrix
ontogenesis
X-linked hypophosphatemia
in situ hybridization
Northern blot analysis

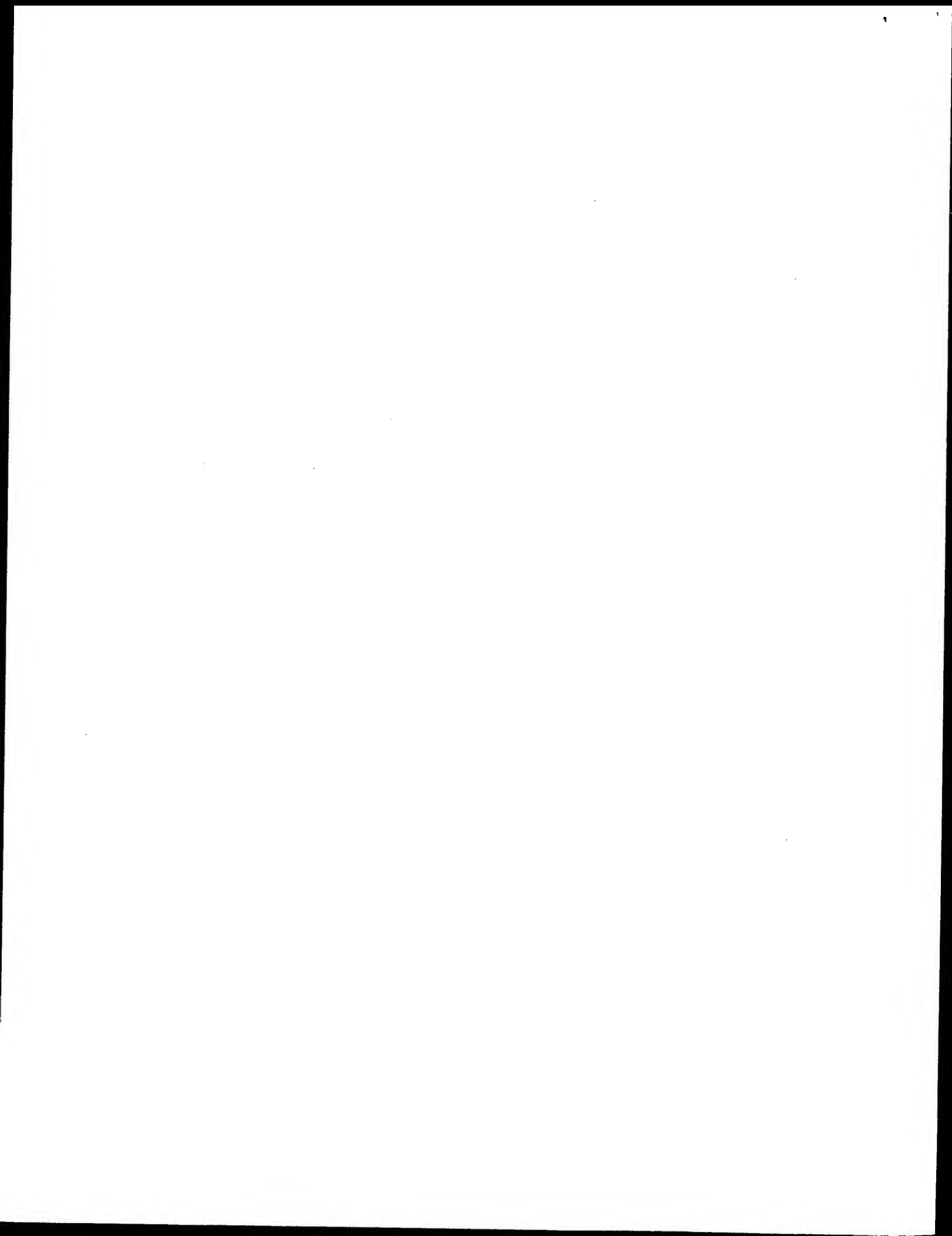
A POSITIONAL CLONING approach was recently used to identify PEX (Phosphate regulating gene with homologies to Endopeptidases on the X chromosome) as the candidate gene for X-linked hypophosphatemia (XLH) (The HYP Consortium 1995). XLH is a Mendelian disorder of phosphate homeostasis characterized by growth retardation, rachitic and osteomalacic bone disease, hypophosphatemia, and renal defects in phosphate reabsorption and vitamin D metabolism (Rasmussen and Tenenhouse 1995). Two mouse models, Hyp (Eicher et al. 1976) and Gy (Lyon et al. 1986), with phenotypic features that are similar to XLH pa-

tients have been described. In the Hyp mouse, the Pex gene harbors a deletion of its 3' end (Beck et al. 1997; Strom et al. 1997), whereas in the Gy mouse the promoter region and the first three exons are deleted (Strom et al. 1997). Studies of these mutant mice contributed greatly to our present understanding of the pathophysiology of the human disease.

Human and mouse PEX/Pex cDNAs have now been cloned and sequenced (Du et al. 1996; Beck et al. 1997; Guo and Quarles 1997; Strom et al. 1997). Amino acid sequence comparisons have demonstrated structural homologies between PEX/Pex and members of the neutral endopeptidase family as previously observed in the partial sequence of the candidate gene (The HYP Consortium 1995). The peptidases of the neutral endopeptidase family are Type II integral membrane glycoproteins with a relatively short cytoplasmic N-termi-

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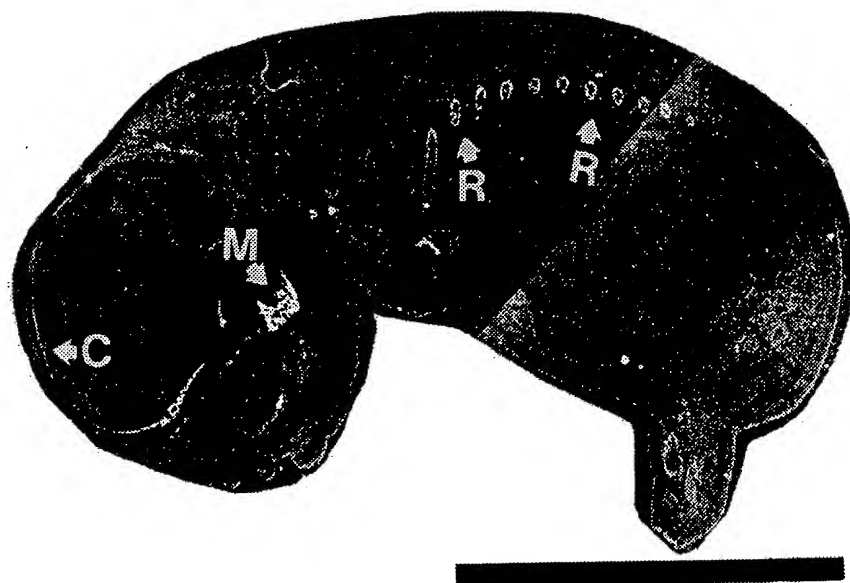


Figure 1 Emulsion autoradiography showing ISH pattern for Pex mRNA at the anatomic level in a sagittal section from a mouse embryo at e16. A significant concentration of Pex mRNA is seen in developing bones (arrows) including calvaria (C), mandible (M), and ribs (R). Bar = 1 cm.

nal region, a single transmembrane domain, and a long extracytoplasmic domain, which contains the active site of the enzyme. Known members of the neutral endopeptidase family include neutral endopeptidase-24.11 (NEP), endothelin-converting enzymes (ECEs), and the erythrocyte cell surface protein KELL (for a review see Turner and Tanzawa 1997). NEP [also known as nepilysin, common acute lymphoblastic leukemia antigen (CALLA), CD10, or enkephalinase] is a widely distributed peptidase involved in the degradation of several bioactive peptides, such as the enkephalins, the atrial natriuretic peptides, and the endothelins (Turner and Tanzawa 1997). The ECEs are involved in the bioactivation of Big endothelins into endothelins, but no function has yet been attributed to KELL.

The mechanism by which loss of PEX function elicits the bone and renal abnormalities observed in XLH patients is not clear. There are no data suggesting the presence of PEX/Pex mRNA in the kidney (Du et al. 1996; Beck et al. 1997; Grieff et al. 1997). In contrast, PEX/Pex mRNA was detected in bones by Northern blot hybridization and in other adult and fetal tissues, such as lungs, liver, muscles, and ovaries by RT-PCR and RNase protection assays (Du et al. 1996; Beck et al. 1997).

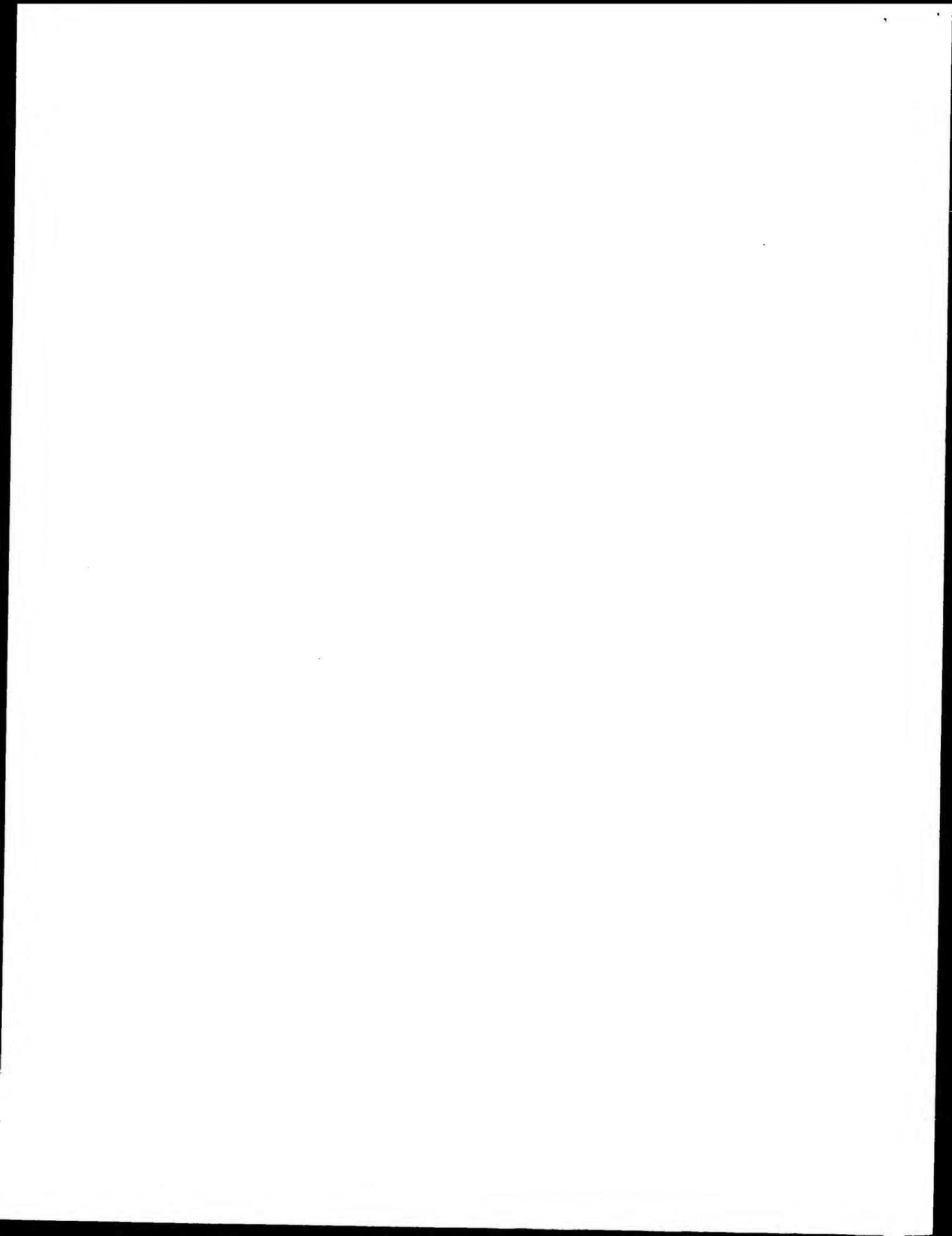
To identify a specific role for PEX/Pex, we were interested first in its tissue and cell distribution. For this reason, using ISH we examined Pex mRNA temporal and spatial patterns of expression on sagittal sections of embryonic mice from Day 13 to 19 and in newborn mice. At this period of development, most tissues are already formed and many are functional. For example, the ossification process starts on Day 14.5 post coitum (Rugh 1991). We demonstrate by ISH that Pex mRNA is expressed in osteoblasts and odontoblasts and suggest a specific role for this putative peptidase in bone and tooth development. Northern analysis was used to examine the presence of Pex mRNA in adult mouse tissues. This analysis revealed decreased concentrations of Pex mRNA in the adult bones and non-growing teeth.

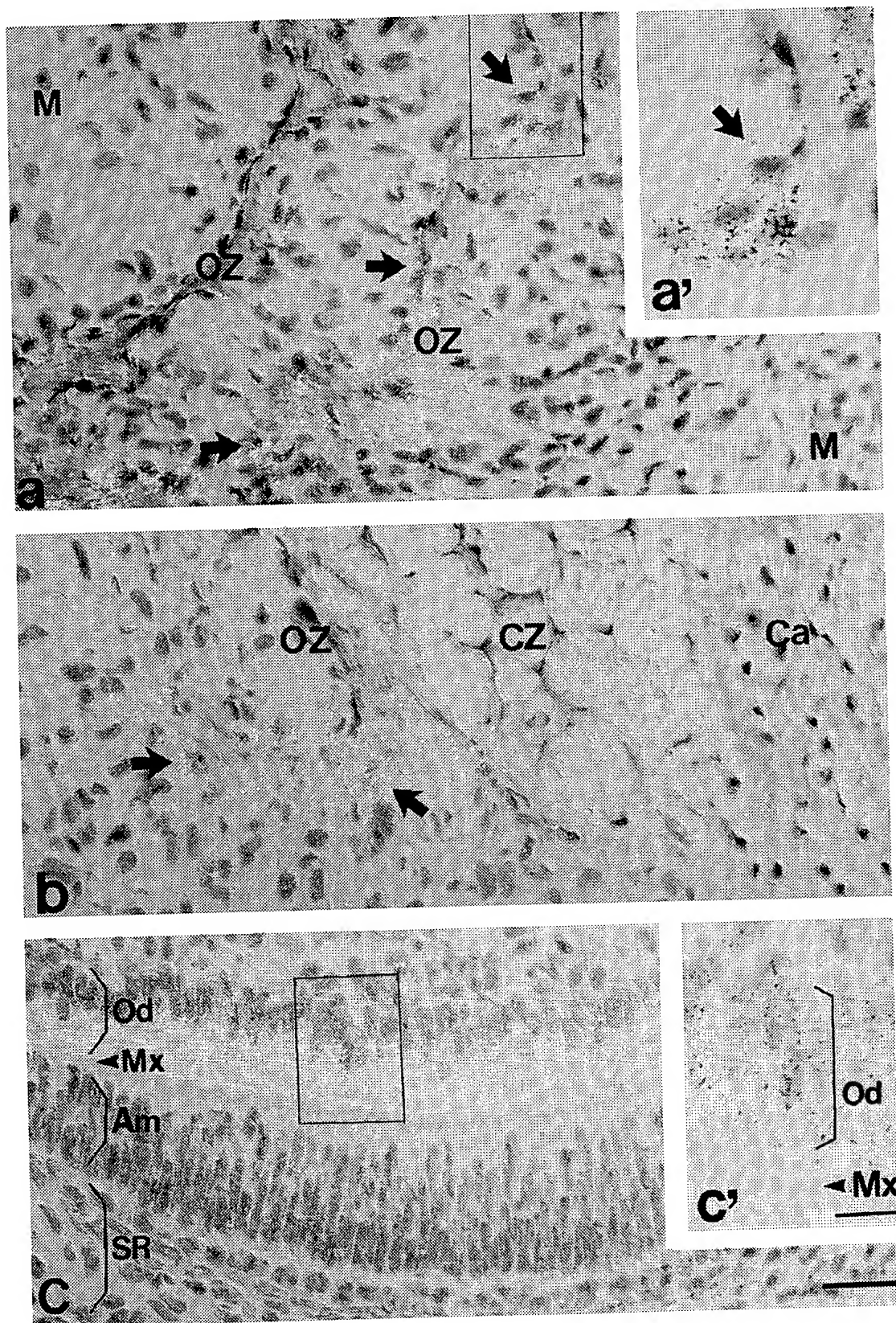
Materials and Methods

Animals

For ISH, we used unfixed, frozen tissues from fetuses of CD1 time-pregnant female mice. The fetuses were grouped according to embryonic age—10, 11, 12, 13, 14, 15, 16, 17, 18 and 19 intrauterine days of life (e10, e11, etc.) and post-natal Day 3 and 7 (p3 and p7) and were prepared as de-

Figure 2 Sites of Pex mRNA expression in primordium of mandibular bone (a) and vertebrae (b) at day e16, and incisor at e19 (c), at a cellular level seen under Nomarski's optics. Silver labeling is seen as white spots (or dark in a' and c') on a color background. In the mandibular bone primordium (a), Pex mRNA is detected in the osteoblasts (arrows) present in the intraconjunctive ossification zone (OZ) surrounded by unlabeled mesenchymal cells (M). A group of osteoblasts is shown at higher magnification in a'. The presence of Pex mRNA (arrows) in vertebrae within endochondral ossification zone (OZ) is shown in b. Note unlabeled adjacent calcified zone (CZ) and cartilage (Ca). (c) A layer of labeled odontoblasts (Od) is shown. A group of odontoblasts is shown at higher magnification in c'. This layer is separated from a layer of unlabeled ameloblasts (Am) by the dentino-enamel matrix (Mx). The stellate reticulum (SR) of enamel organ is also unlabeled. Bars: a-c = 25 μ m; a'-c' = 10 μ m.





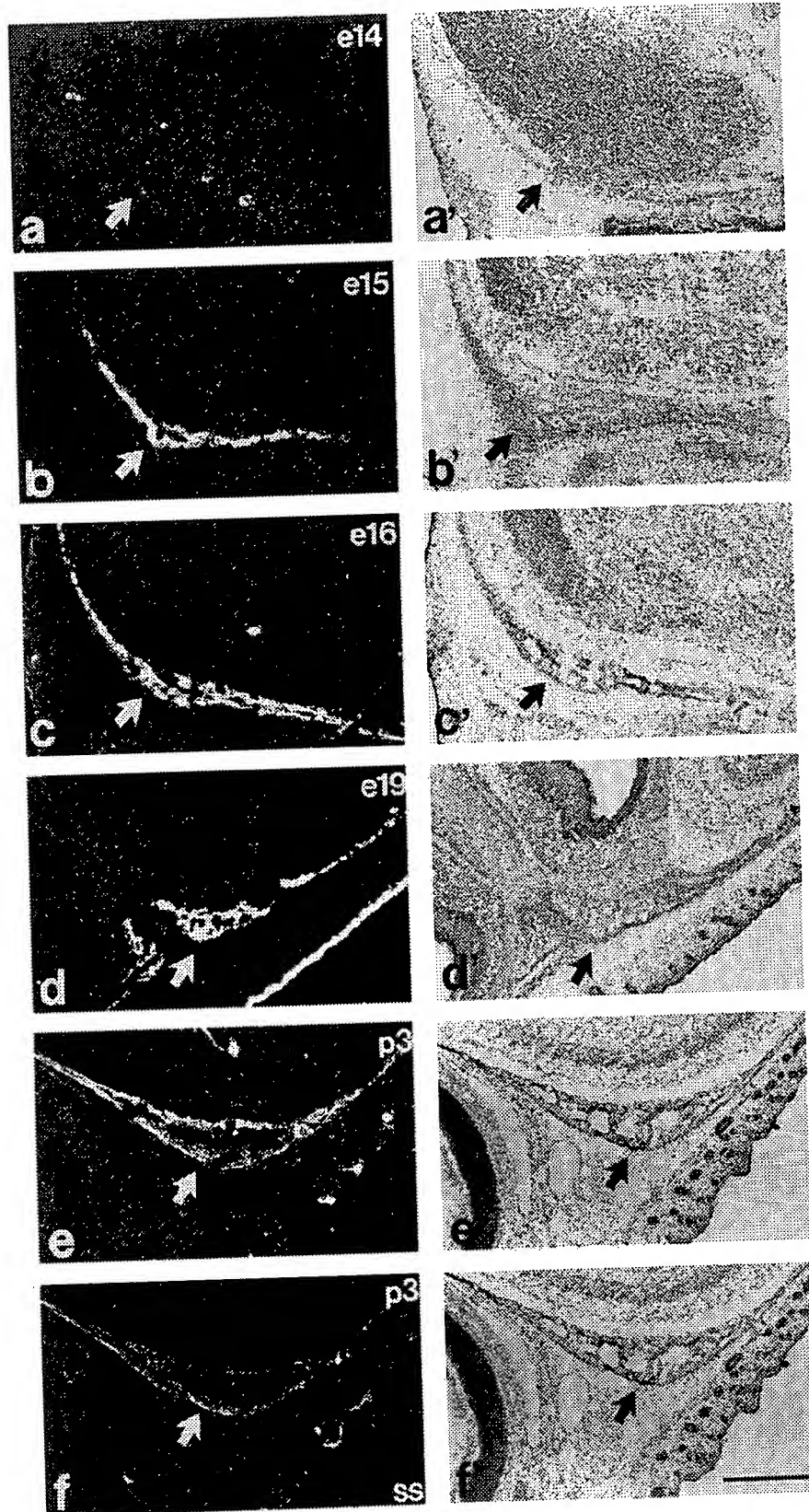
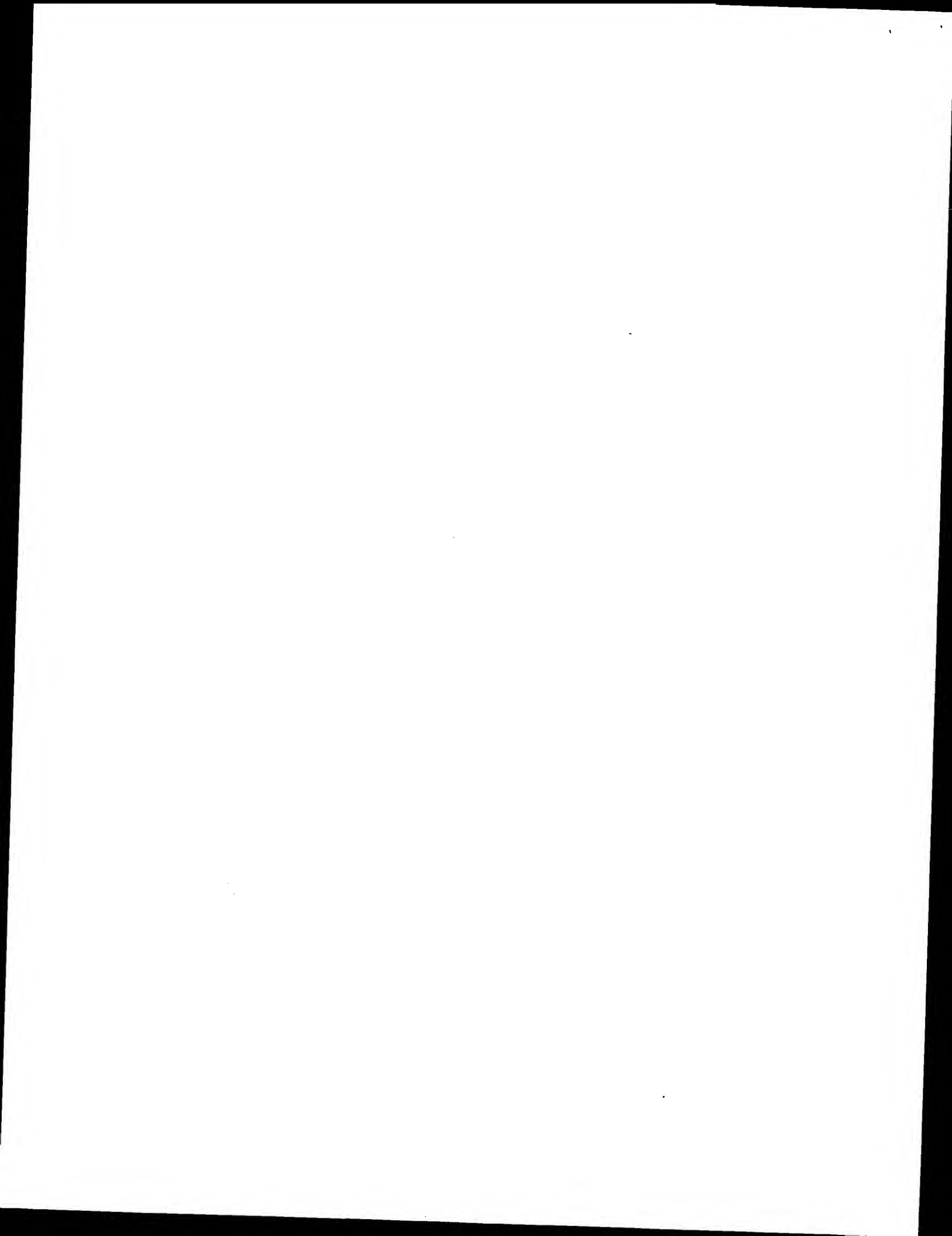


Figure 3 Sites of Pex mRNA expression in developing mouse calvaria on e14 (a), e15 (b), e16 (c), e19 (d), and p3 (e). Labeling is seen as white spots on darkfield (arrows in a-f) and as black labeling on brightfield (arrows in a'-f'). (f) Control hybridization obtained with sense riboprobes. Bar = 500 μ m.



scribed (Marcinkiewicz et al. 1993). Whole embryos and newborn mice were rapidly removed, cooled in ice-cold PBS, embedded in Tissue-Tek OCT compound (Miles; Elkhart, IN), frozen at -30°C in isopentane, and cut into $10\text{-}\mu\text{m}$ sections. The sections were mounted on 0.5% gelatin-coated slides and stored at -80°C .

DNA Manipulations

All DNA manipulations, including the reverse transcription from RNA, PCR, and cloning, were done according to standard protocols (Sambrook et al. 1989; Ausubel et al. 1994). DNA sequencing was performed on double-stranded DNA (Tabor and Richardson 1987).

Cloning of a Mouse Partial Pex cDNA

To obtain a mouse Pex probe for ISH and Northern blot analysis, degenerate oligonucleotides corresponding to conserved regions among members of the neutral endopeptidase family were designed using the published human PEX gene sequence (The HYP Consortium 1995) and were used in an RT-nested PCR reaction with polyA⁺ RNA purified from testis of CD1 mice (Charles River; Montréal, Québec, Canada). The expected 337-bp DNA fragment was generated and cloned in plasmid pCRII (Invitrogen; Carlsbad, CA). Sequencing of the DNA insert showed more than 97% identity with the human PEX gene sequence (The HYP Consortium 1995) and it is identical to a DNA stretch spanning the region between asparagine 454 and proline 566 of the published cDNA sequence of mouse Pex (Du et al. 1996; Beck et al. 1997; Strom et al. 1997).

Preparation of cRNA Probes

The pCRII plasmid containing the Pex cDNA fragment was linearized with XhoI and used as a template in an *in vitro* transcription assay to synthesize a single-stranded anti-sense RNA probe with SP6 RNA polymerase. For control, sense RNA probe was synthesized with T7 RNA polymerase after linearization of the plasmid with KpnI. For ISH, Pex riboprobes were labeled with both [³⁵S]-UTP and [³⁵S]-CTP (1250 Ci/mmol; Amersham, Arlington Heights, IL) because very low mRNA levels were reported previously (The HYP Consortium 1995; Du et al. 1996; Beck et al. 1997). For Northern blot analysis, probes were labeled with [³²P]-UTP (800 Ci/mmol; Dupont/NEN, Wilmington, DE). The 18S rRNA probe (a generous gift from Dr. M. Uhler) is of bovine origin and strongly cross-hybridizes to a number of different mammalian species.

In Situ Hybridization

ISH was undertaken using RNase-free solutions, starting with frozen cryostat tissue sections that were slowly immersed in cold formaldehyde in 0.1 M phosphate buffer (pH 7.2) and maintained in this solution for 45–60 min, then washed extensively with PBS. The tissues were treated for 10 min with acetic anhydride in 0.1 M TEA. After dehydration with alcohol, the tissue sections were dried and then incubated overnight at 55°C with a hybridization solution consisting of 75% formamide, 10% polyethylene glycol, $3 \times \text{SSC}$ ($1 \times \text{SSC} = 0.15 \text{ M NaCl}$, $0.015 \text{ M Na citrate}$, pH 7.2),

50 mM phosphate buffer, pH 7.2, $1 \times \text{Denhardt's}$ (made from $50 \times$ stock solution: 1% Ficoll, 1% polyvinylpyrrolidone, and 1% bovine serum albumin in water), 0.5 mg/ml yeast tRNA, and 0.1 mg/ml sonicated denatured salmon sperm DNA. To increase the signal/noise ratio, the dithiothreitol (DTT) concentration was set at 200 mM (Miller et al. 1993). After hybridization, the sections were washed sequentially in $2 \times$, $1 \times$, $0.5 \times$, and $0.1 \times \text{SSC}$ containing 10 mM DTT for 10, 15, 20 and 60 min at 20°C , 20°C , 50°C , and 55°C , respectively. After the washing step in $1 \times \text{SSC}$, the sections were incubated with RNase A at 200 $\mu\text{g/ml}$ for 30 min at 37°C to remove unbound cRNAs. Sections were then dehydrated in a series of alcohol baths and dried. Hybridization was examined on X-ray film (exposure time 5 days), followed by autoradiography using NTB-2 emulsion (Kodak; Rochester, NY) for 30 days at 4°C and development in D19 solution (Kodak). The sections were stained with hematoxylin-eosin and viewed under dark- and brightfield illumination. Some observations were done using Nomarski's attachment.

Alkaline Phosphatase Activity

Localization of alkaline phosphatase activity (Roach and Shearer 1989) was performed with an azo dye coupling method (Alkaline Phosphatase Substrate Kit, cat. # SK-5300 Vector Blue) as recommended by the manufacturer (Vector Laboratories; Burlingame, CA).

RNA Extraction and Northern Blot Analysis

Northern blot analysis was performed using total RNA from newborn and adult calvariae and teeth, and from newborn brain, lung, and liver. For this purpose, the frozen tissues were mixed with TRIzol Reagent (Life Technologies/Gibco-BRL; Burlington, Ontario, Canada) and disrupted with a Polytron. Total RNA was extracted according to Chomczynski (1993), as recommended by the manufacturer. As estimated by spectroscopy at 260 nm, 16 μg of total RNA samples was loaded per lane in 1% agarose gel containing 20 mM HEPES, pH 7.8, 1 mM EDTA, and 6% formaldehyde. After electrophoresis, the RNA was transferred from the gel to a nylon membrane and fixed to the filters by long-wave UV irradiation. The filters were prehybridized at 65°C for 90 min in a solution composed of 5% SDS, NaPO_4 0.4 M, pH 7.2, EDTA 1 mM, 0.1% BSA, 50% formamide (Gibco/BRL) and were hybridized overnight at 65°C in the presence of the [³²P]-UTP-labeled cRNA probe. After hybridization, the filters were washed in $0.1 \times \text{SSC}$, 0.1% SDS, 1 mM EDTA at 70°C for 2 hr and exposed at -70°C for 6 days to X-ray film (Kodak) with an intensifying screen. Once the exposure step was completed, the blots were boiled in 0.1% SDS, 1 mM EDTA for 10–15 min and re-probed with 18S rRNA.

Results

Pex ISH at Anatomic Resolution in Mouse Embryo

The Pex expression pattern was analyzed by ISH using anti-sense riboprobes on histological sections obtained from embryonic and postnatal mice (Figures 1–5). ISH at anatomic resolution was examined after emulsion autoradiography on embryonic Day 16 (e16) (Figure

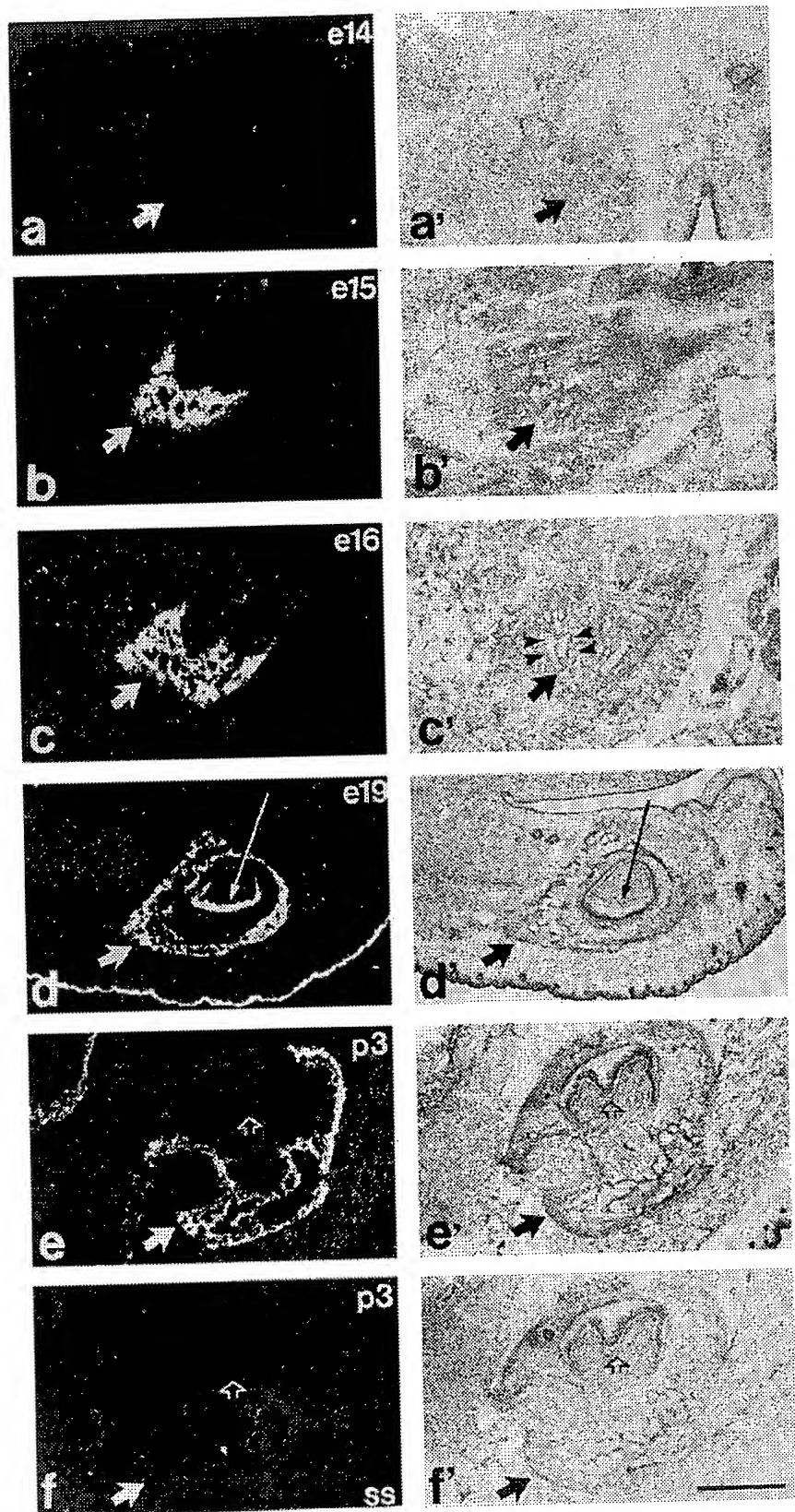


Figure 4 Sites of Pex mRNA expression in mandible bone on e14 (a), e15 (b), e16 (c), e19 (d), and p3 (e) (short arrows). The presence of Pex mRNA is also evident in developing incisor (long arrows) on e19 (d). A second molar at an early stage of development is unmarked (open arrow in e). The same sections are shown after hematoxylin-eosin staining (a'-f'). Area delineated by four arrowheads in c' is shown at higher magnification in Figure 2a. (f) Control hybridization obtained with sense riboprobes. Bar = 500 μ m.

1). Pex mRNA was readily detectable in regions of calvaria, mandible, and ribs. Although not apparent in Figure 1, the vertebrae and long bones also contained Pex mRNA on e16, whereas later, around birth, Pex mRNA could also be detected in developing teeth. Overall, Pex mRNA was localized within alkaline phosphatase territory (not shown). Controls were performed with sense riboprobes, which produced nonspecific background elevated in skin (see also Figures 3d and 4d) and skeletal muscles (not shown). Other tissues, including kidney, lung, liver, and brain, were negative.

Pex mRNA in Osteoblasts

To identify cells synthesizing Pex mRNA, representative tissues were examined under higher microscopic magnification. Figures 2a and 2a', which are higher magnifications of the area shown in Figure 4c, demonstrate the presence of Pex mRNA on e16 in mandibular intramembraneous ossification centers. This topographically heterogeneous region is composed of an ossification zone (OZ) characterized by the presence of (a) a bone extracellular matrix with a dense cell population on the border zone and (b) an undifferentiated mesenchyme (M). ISH revealed the presence of Pex mRNA within cells bordering bone extracellular matrix. This topography suggests that Pex-expressing cells are osteoblasts. The majority of osteoblasts display Pex ISH labeling (Figure 2a').

Hybridization sites were also identified in vertebral endochondral ossification centers (Figure 2b). Pex mRNA was seen within an ossification zone (OZ) adjacent to an unlabeled calcified zone (CZ) and the cartilage (Ca). Bone extracellular matrix was well delineated within OZ, with a significant concentration of Pex-labeled cells on a border zone. There was no apparent hybridization labeling outside of OZ.

Pex mRNA in Odontoblasts

Within tooth, Pex mRNA was concentrated in the layer of odontoblasts (Od) (Figure 2c, which represents a higher magnification of the area shown in Figures 5b and 2c'). In contrast, the layer of ameloblasts (Am) and the stellate reticulum of the enamel organ (SR) were unlabeled. Because the dentino-enamel matrix separates the odontoblast epithelium from the ameloblast epithelial layer, the cell population expressing Pex mRNA is particularly well-defined in this tissue.

Pex mRNA Ontogeny

To determine temporal and tissue-specific patterns of Pex gene expression, the calvaria, mandible, and teeth were examined at the stages preceding and following the onset of chondrification (e11), ossification (e14.5), and odontogenesis (e14). ISH results are shown in Figures 3-5.

Figure 3 shows the presence of Pex mRNA in the calvaria from e15 to p3. Although rudimentary calvaria is already visible on e14 (Figures 3a and 3a'), Pex mRNA is not detectable at this time but is evident thereafter. It is worth noting the presence of bone extracellular matrix within rudimentary calvaria at e15 but not e14. This matrix was seen as a deposition of acellular eosinophilic material along and within a space delineated by a front of osteoblasts (data not shown). Pex mRNA remains present in the calvaria during later gestation and early postnatal development.

Figure 4 depicts Pex mRNA distribution in the mandible from e15 to p3. Despite the absence of the bone extracellular matrix, which is not present early on, the rudiments of the mandible bone are evident by e14. Similar to calvaria, a dramatic elevation of Pex mRNA is evident on e15 (Figure 4b). Pex mRNA remains present until p3.

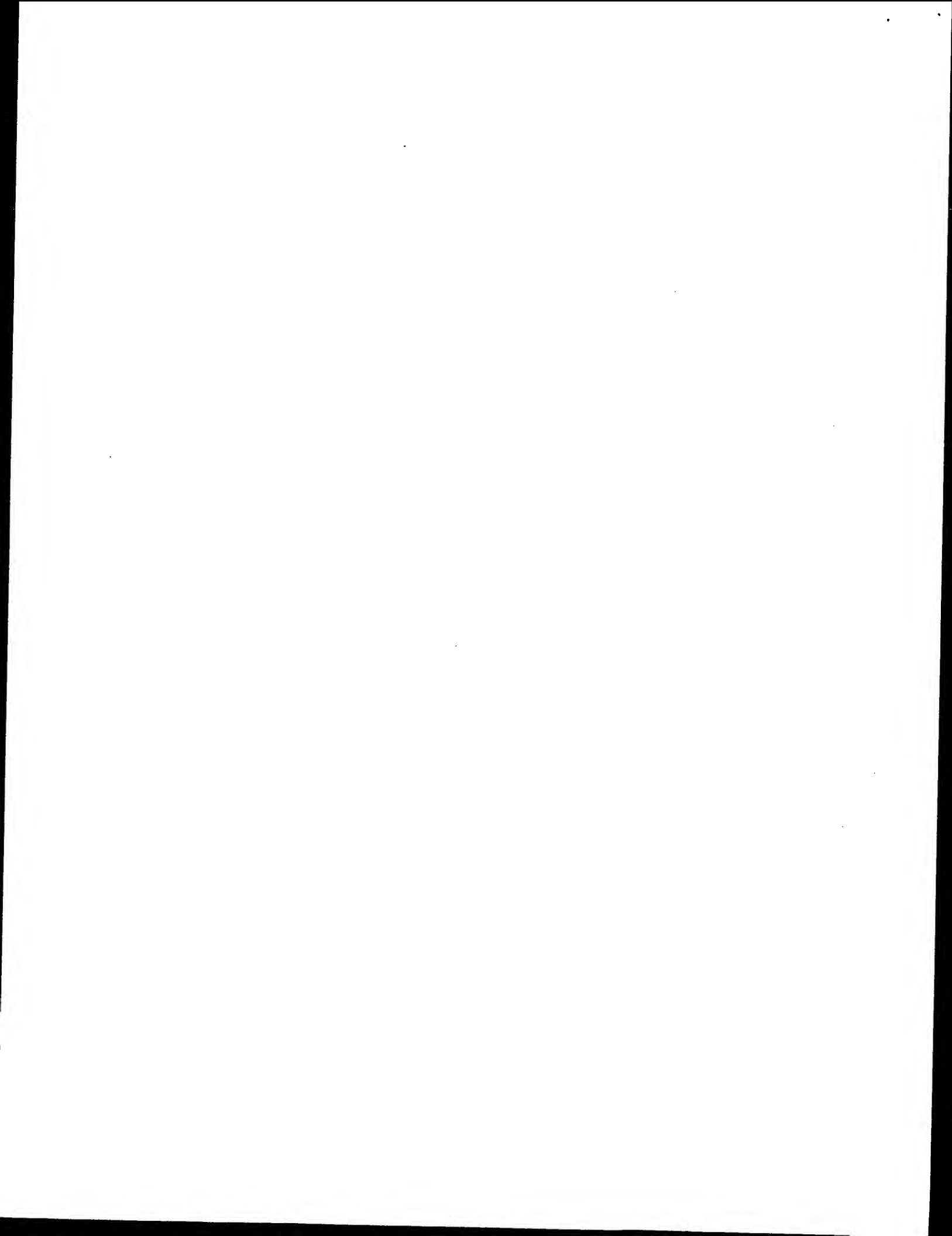
In addition to bones, Pex mRNA labeling can be observed in both incisors and molars, although the onset of expression varies with time of tooth development (Figures 4 and 5). All teeth shown in Figure 5 are from the same section of e19 mouse and include one molar (Figures 5a and 5a'), one inferior incisor (Figures 5b and 5b'), and one superior incisor (Figures 5c and 5c'). Incisors are strongly labeled, whereas rudimentary molars not. The presence of dental extracellular matrix was noted within the two incisors but not in the molar. In addition to incisors, first molars showed PEX mRNA labeling on p3, whereas second molars were positive on p7 (results not shown).

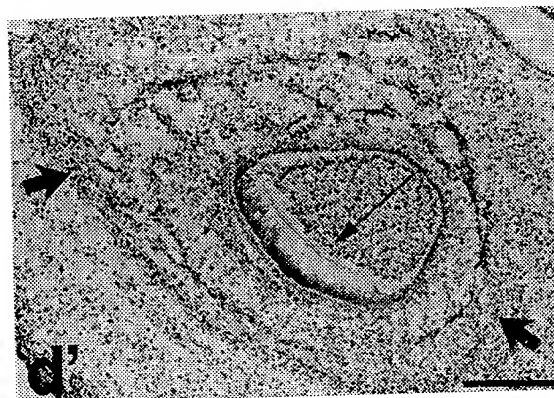
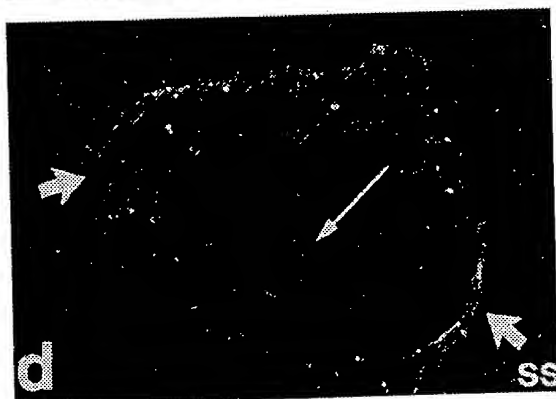
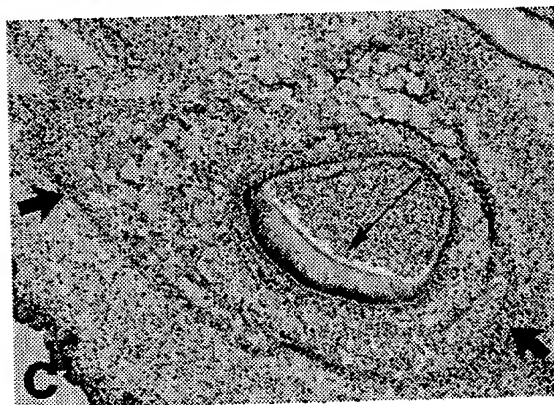
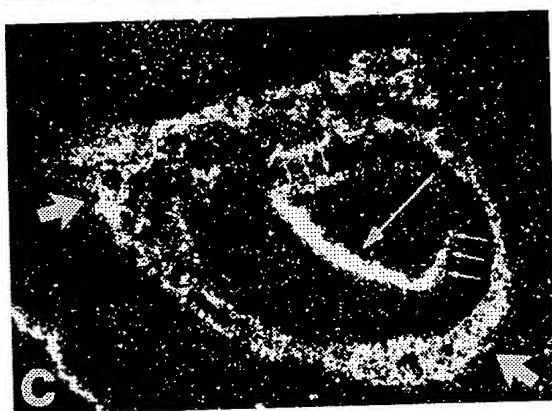
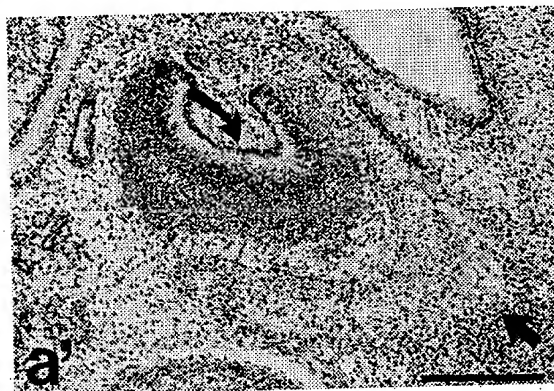
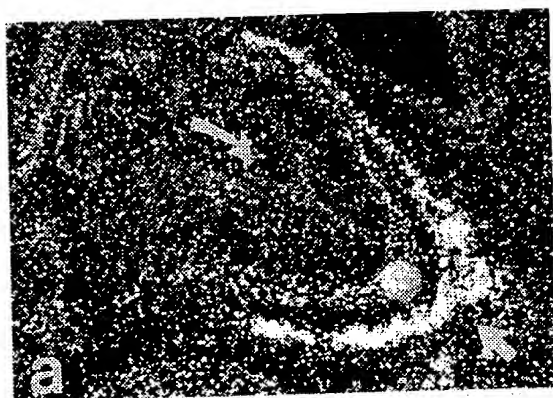
Characterization of Pex mRNA in Adult Bone and Teeth

To verify the presence of Pex mRNA in adult mouse, Northern analysis was performed with total RNA obtained from p3 and adult tissues. A Pex transcript of approximately 7 kb was detected in calvariae and teeth (Figure 6). With 18S RNA as an internal control for mRNA loading, it is clear that PEX mRNA levels in calvariae are higher on p3 than in adulthood. Pex mRNA was observed in p3 and adult teeth. Knowing that incisors, but not molars, present continuous growth in rodents, we isolated total RNA from adult incisors and molars and compared their levels of Pex mRNA. This comparison demonstrated that adult incisors express higher mRNA levels of Pex than adult molars. Pex mRNA was not detectable in newborn lungs, liver, and brain by Northern analysis.

Discussion

This report provides histochemical evidence for Pex mRNA expression in murine embryonic and postnatal bones and teeth. In these tissues, Pex mRNA was de-





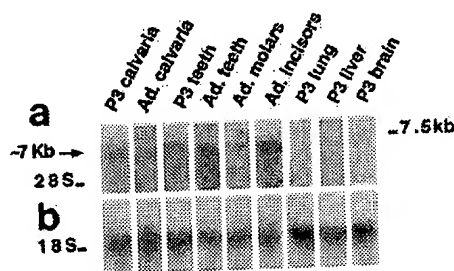


Figure 6 Northern blot analysis of Pex mRNA levels in mouse p3 and adult calvariae, teeth, adult molars, and incisors, p3 lung, p3 liver, and p3 brain. Data shown are from three different Northern blot experiments (Lanes 1–3, 4–6, and 7–9). (a) About 16 μ g total RNA/lane was hybridized with a [32 P]-UTP-labeled mouse Pex cRNA probe. A 7-kb transcript (arrow) was identified in calvariae and teeth. Pex mRNA was undetectable in p3 mouse lung, liver and brain. (b) 18S ribosomal RNA shown after hybridization with 18S cRNA probe.

tected in osteoblasts and odontoblasts, respectively. With both ISH and Northern analysis, bones and teeth were the only tissues in which the presence of the Pex mRNA could be detected, suggesting that these two tissues are privileged sites for Pex expression in the developing mouse. A significant concentration of Pex mRNA was also detected in adult incisors, which grow continuously in rodents, whereas in adult calvariae and in nongrowing molars Pex mRNA expression appeared to be considerably lower. Taken together, these data suggest a role for Pex in the development of bones and teeth.

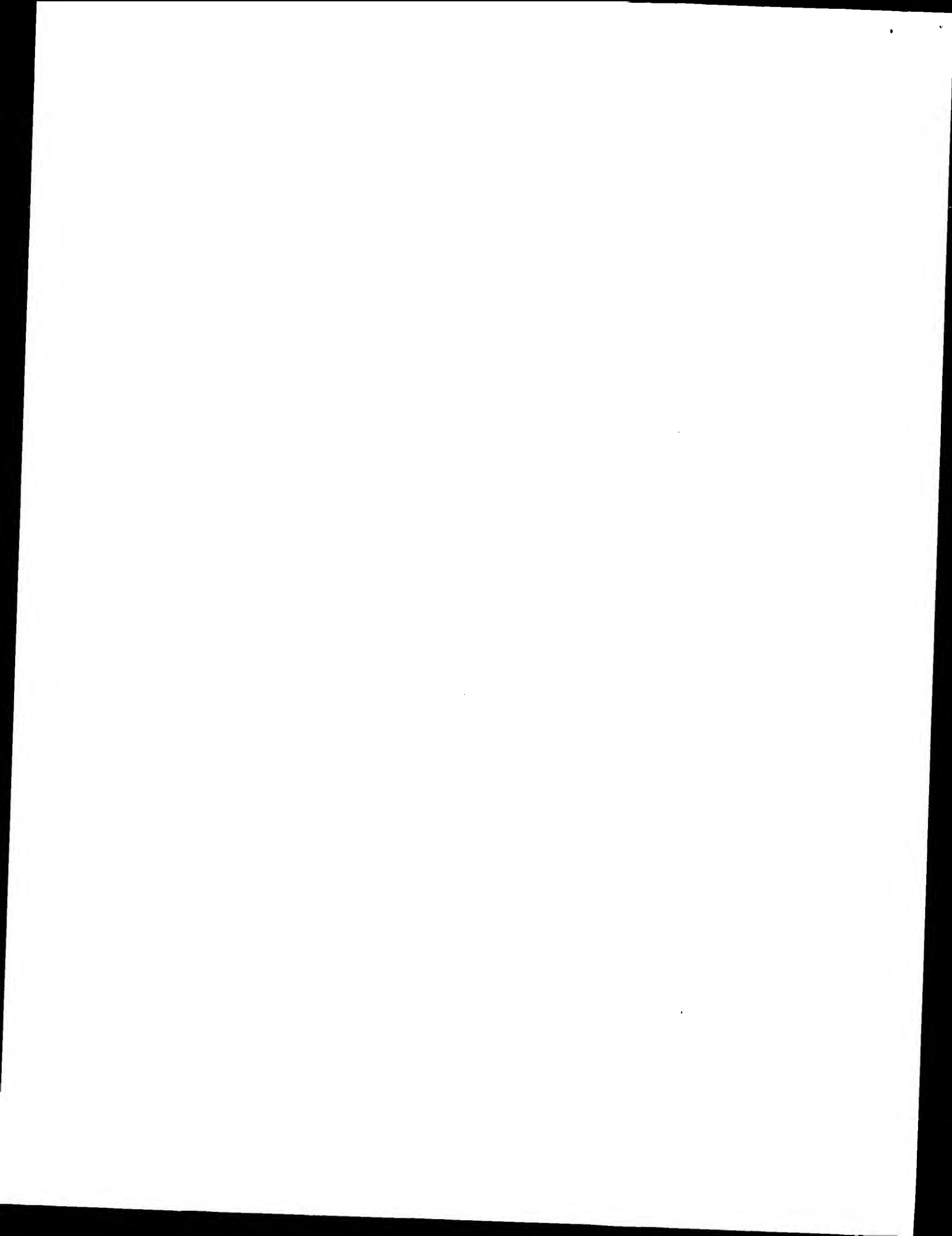
Osteoblasts and chondroblasts/chondrocytes are important cell components of developing bone. Major products of osteoblasts and chondrocytes are the extracellular matrix of bone and cartilage, respectively. The bone extracellular matrix is composed of 90% collagen and 10% noncollagenous proteins (Desbois and Karsenty 1995; Nefussi et al 1997), including osteocalcin, osteopontin, bone sialoprotein, and osteonectin. Our ISH results provide evidence for the presence of Pex mRNA in osteoblasts, localized in close proximity (border zone) to the extracellular matrix, suggesting that Pex may play a role in matrix elaboration, deposition, and/or mineralization. In addition, we demonstrate a temporal relationship between the onset of Pex mRNA expression and the onset of matrix deposition. Although not identical to the situation observed during bone development, Pex mRNA expression in

teeth also suggests a striking correlation with matrix deposition.

Our results are in concordance with the clinical features of Hyp and Gy mice, which harbor large deletions in the Pex gene (Beck et al. 1997; Strom et al. 1997). Both mutant animal models exhibit abnormalities in bone and tooth formation, including rickets, osteomalacia, and formation of an interglobular dentin (Eicher et al. 1976; Lyon et al. 1986; Abe et al. 1992). In Hyp mice, mineralization of bone extracellular matrix is delayed (Ecarot-Charrier et al. 1988; Ecarot et al. 1992). Therefore, loss of Pex function appears to be responsible for the bone mineralization defect in Hyp mice. The precise mechanism by which Pex may regulate mineralization of the extracellular matrix is unclear. However, the Pex protein structure suggests that it may function as a peptidase (The HYP Consortium 1995; Turner and Tanzawa 1997). Therefore, one can postulate that Pex is a key component controlling the bioactivity of one or several peptides influencing osteoblast and odontoblast proliferation and/or differentiation. It may also be involved in proteolysis of extracellular matrix proteins. In this regard, an interesting candidate is osteocalcin, a 46 amino-acid peptide produced and secreted exclusively by osteoblasts and odontoblasts (Desbois et al. 1994; Desbois and Karsenty 1995). Studies performed with knockout mice suggest that osteocalcin interferes with the mineralization process (Ducy et al. 1996), a finding compatible with the plasma levels of osteocalcin in Hyp mice relative to normal mice (Gundberg et al. 1992).

Like patients with XLH, both Hyp and Gy mutant mice develop hypophosphatemia secondary to impaired renal phosphate reabsorption (Rasmussen and Tenenhouse 1995). The relationship between the renal phosphate transport defect in XLH, Hyp, and Gy mice and the loss of Pex function is not clear. A possible role of PEX/Pex may be to metabolize a heretofore unidentified circulating peptide hormone involved in regulation of renal phosphate transport and phosphate homeostasis. A factor that inhibits phosphate uptake by normal mouse proximal tubule cells in primary culture was recently reported in Hyp mouse serum (Lajeunesse et al. 1996), a finding consistent with data from parabiosis experiments between normal and Hyp mice (Meyer et al. 1989) and kidney cross-transplantation studies in Hyp vs normal mice (Nesbitt et al. 1992). This hypothetical phosphaturic factor has not

Figure 5 Pex mRNA present in developing teeth (long arrows) and mandibular bone (short arrows) in e19 mouse. Early (a), medium (b), and more advanced (c) tooth developmental stages correlate with different levels of Pex mRNA, being undetectable in molar primordium (curved arrow in a) and more extended in incisors (b,c). Note lateral extension of Pex mRNA distribution in the more developed incisor (small, thin arrows in c). (d) Control hybridization obtained with sense riboprobes. Area delineated by four arrowheads in b' is shown at higher magnification in Figure 2c. Bar = 200 μ m.



yet been identified and its source has not been established. We suggest that osteoblasts and odontoblasts may be involved in the inactivation of this hypothetical factor. Alternatively, Pex could be involved in the processing of an inactive precursor into a bioactive peptide that stimulates phosphate reabsorption by the kidney.

In conclusion, we demonstrate the presence of Pex mRNA in both embryonic and adult bones and teeth. Cells expressing Pex have been identified as osteoblasts and odontoblasts. Therefore, Pex could be a useful marker for these two cell types. In adult bones, the levels of Pex mRNA were lower than those in embryos. In adult teeth, Pex mRNA concentration remained elevated in incisors and was lower in molars. These results suggest that Pex is involved in the development of bone and tooth tissues.

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SUMMARY LANGUAGE: Japanese; English ANSWER 17 OF 29 EMBASE COPYRIGHT 2002 ELSEVIER SCI.
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AUTHOR: Murer H.; Forster I.; Hilfiker H.; Pfister M.; Kaissling B.; Lotscher M.; Biber J.
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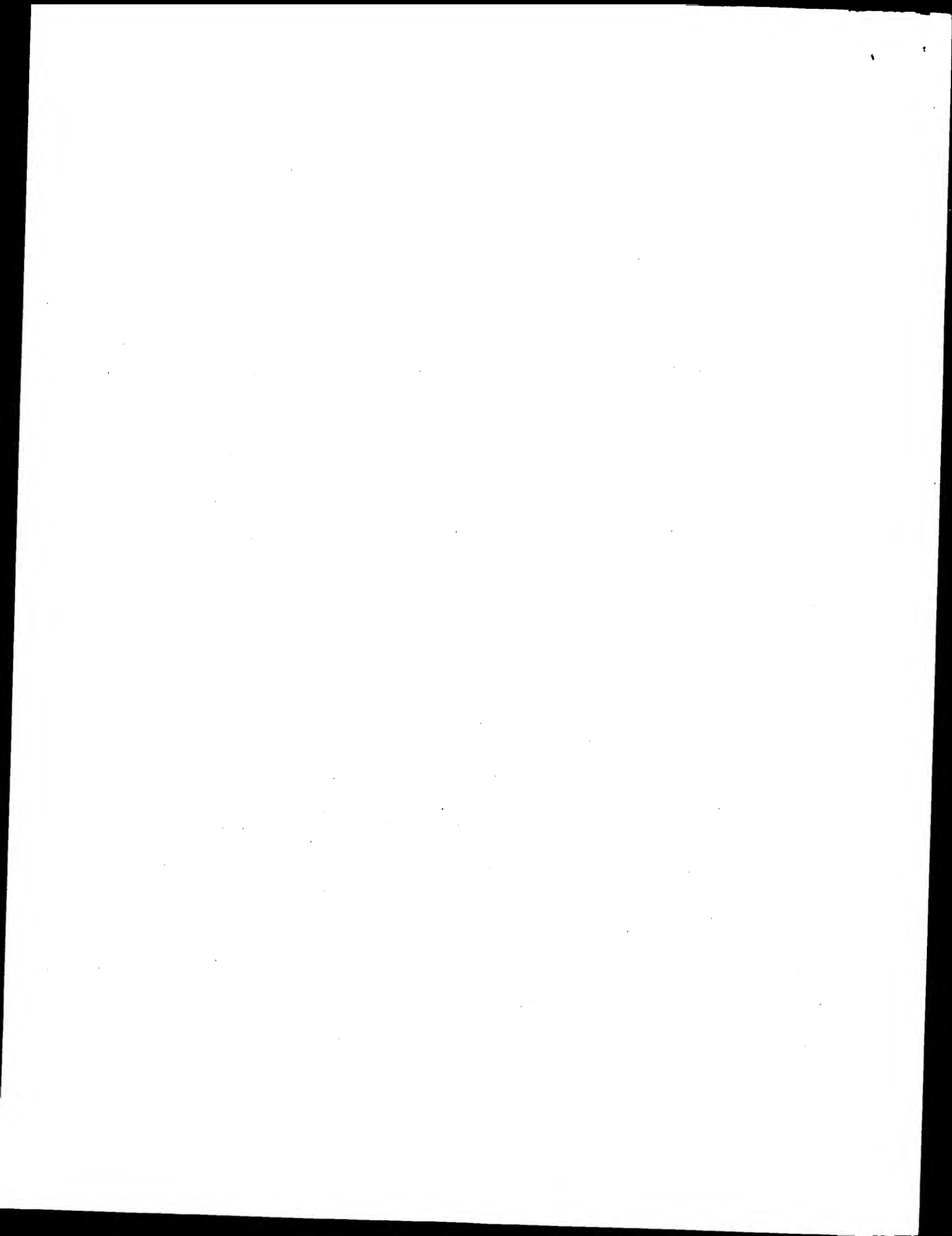
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AUTHOR: Beck L.; Soumounou Y.; Martel J.; Krishnamurthy G.; Gauthier C.; Goodyer C.G.; Tenenhouse H.S.
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AUTHOR: Nelson A.E.; Robinson B.G.; Mason R.S.
CORPORATE SOURCE: Dr. A.E. Nelson, Molecular Genetics Department, Kolling Inst. of Medical Research, Royal North Shore Hospital, St Leonards, NSW 2065, Australia. annen@med.usyd.edu.au
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Cellular/molecular control of renal Na/P_i-cotransport

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Cellular/molecular control of renal Na/P_i-cotransport. A type II Na/P_i-cotransporter located in the brush border membrane is the rate limiting and physiologically regulated step in proximal tubular phosphate (P_i) reabsorption. In states of altered P_i-reabsorption [for example, in response to parathyroid hormone (PTH) and to altered dietary intake of P_i or as a consequence of genetic abnormalities], brush border expression of the type II Na/P_i-cotransporter is accordingly modified. PTH initiates a regulatory cascade leading to membrane retrieval, followed by lysosomal degradation of this transporter; recovery from inhibition requires its *de novo* synthesis. P_i-deprivation leads to an increased brush border expression of transporters that does not appear to require *de novo* synthesis in the short term. P_i-overload leads to membrane retrieval and degradation of transporters. Finally, in animals with genetically altered P_i-handling (*Hyp*; *Gy*) the brush border membrane expression of the type II Na/P_i-cotransporter is also reduced, suggesting that a genetically altered protein (such as *PEX* in *Hyp*) controls the expression of this transporter.

Renal proximal tubular P_i-reabsorption is a key component in overall P_i-homeostasis [1, 2]. Different *in vivo* and *in vitro* studies (such as tubular microperfusion and studies with membrane vesicles) suggested that a brush border membrane Na/P_i-cotransporter determines proximal tubular transepithelial transport rate and is therefore also the main target in physiological/pathophysiological altered P_i-reabsorption [reviewed in 2-6]. Recently, different Na/P_i-cotransporters have been structurally identified ('cloned'). Using specific cDNA probes and antibodies it could be documented that altered expression of a brush border membrane type II Na/P_i-cotransporter satisfactorily explains physiologically and pathophysiological altered P_i-reabsorption [reviewed in 7-10].

In this review we briefly summarize the present knowledge of different mammalian Na/P_i-cotransporters, the molecular and functional properties of the renal proximal tubular type II Na/P_i-cotransporter, and the cellular mechanisms involved in the control of altered brush border expression of this transporter.

'CLONING' OF DIFFERENT MAMMALIAN Na/P_i-COTRANSPORTERS

Our laboratory was the first to isolate by expression cloning techniques (*Xenopus laevis* oocytes) cDNAs related to renal Na/P_i-cotransporters. NaPi-1 was isolated from rabbit [11] and NaPi-2 and NaPi-3 were isolated from rat and human kidney

cortex cDNA libraries, respectively [12]. Detailed sequence comparisons indicated that NaPi-1 and NaPi-2/3 are different proteins. Therefore, we have introduced the nomenclature *type I* (NaPi-1) and *type II* Na/P_i-cotransporters (NaPi-2/3) [7, 8]. Homologues of NaPi-1 and therefore type I transporters have been identified in kidneys of different species (rats, rNaPi-1 [13]; humans, NPT-1 [14]; mice, Npt-1 [15]) and in brains of rats (rBNPI [16, 17]) and humans (hBNPI [18]). Homologues of NaPi-2/3 (that is, type II Na/P_i-cotransporters) were isolated from opossum kidney cells (NaPi-4 [19]), from the bovine renal epithelial cell line NBL-1 [20], from flounder kidney and intestine (NaPi-5 [21, 22]), from rabbit kidney (NaPi-6 [23]) and from mouse kidney (NaPi-7 [24, 25]). Two additional Na/P_i-cotransporters have been recently identified and can be classified as type III. Both are high affinity Na/P_i-cotransporters: *Ghr-1*, the receptor for gibbon ape leukemia virus and feline leukemia virus B, and *Ram-1*, the receptor for ectotropic murine leukemia virus. They have been named by the authors as Pit-1 and Pit-2, respectively [26-29].

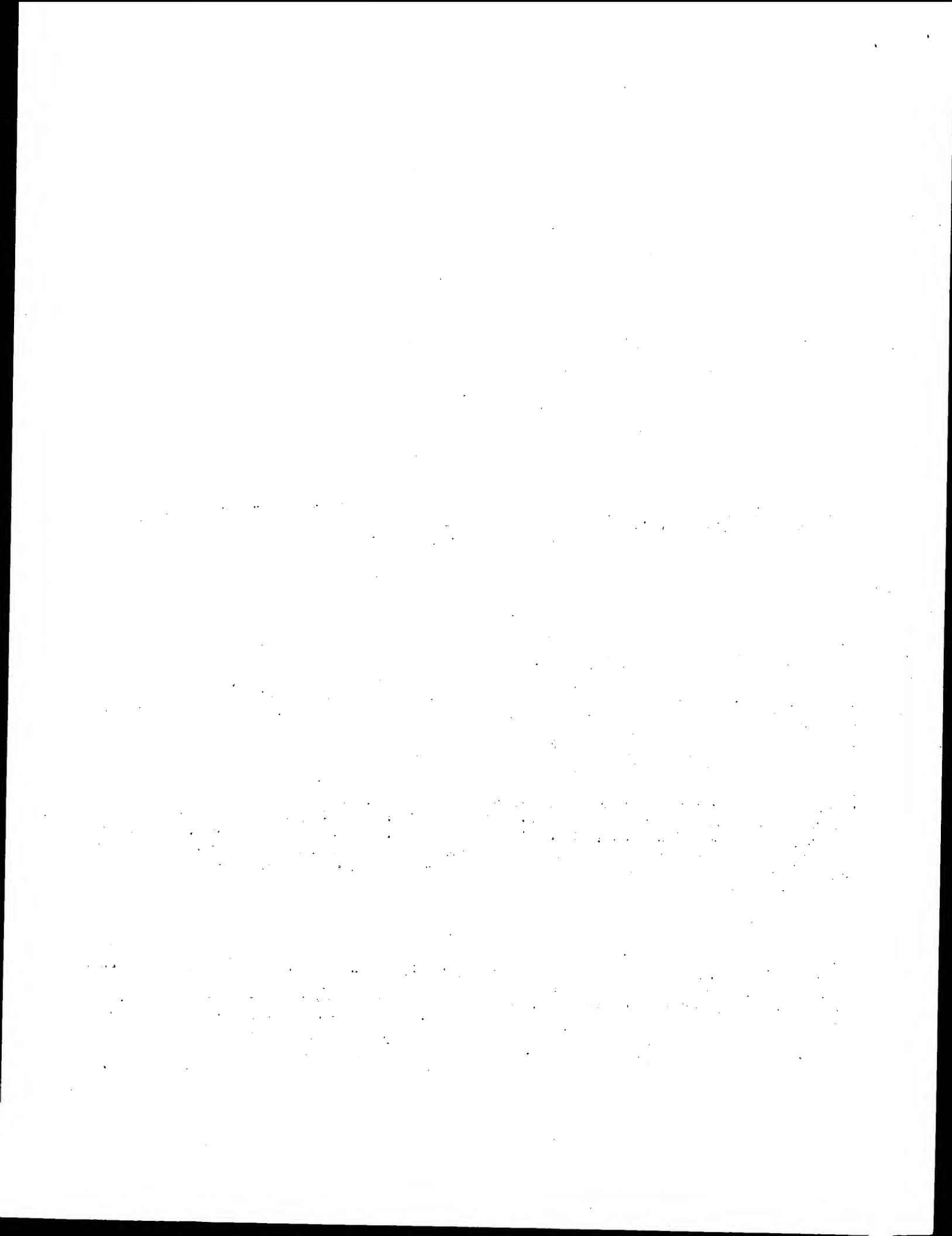
MOLECULAR AND FUNCTIONAL PROPERTIES OF THE TYPE II Na/P_i-COTRANSPORTER

In any expression cloning strategy that starts with mRNAs and cDNA-libraries originating from a heterogenous tissue source, it is important to document the appropriate cellular location of the mRNA and the encoded protein. Pit-1 and Pit-2 (type III Na/P_i-cotransporters) are widely distributed; however, their precise location in the kidney has not been determined [26, 29]. On the other hand, the type I and type II Na/P_i-cotransporters are preferentially located in the kidney. Reverse transcription-polymerase chain reaction (RT-PCR) on microdissected tubules and *in situ* hybridizations indicated an almost exclusive proximal tubular location, and studies using specific antibodies showed proximal tubular brush border location of NaPi-1 and NaPi-2 (for NaPi-2 see Fig. 1) [15, 24, 30-33].

Little information is available on the molecular structure of the Na/P_i-cotransporters; hydropathy analysis suggests the presence of several *trans*-membrane domains, such as at least 10 for Pit-1/2 (type III) [26-29], 6 for NaPi-1 (type I) [7, 8, 11], and 8 for type II (see Fig. 1 for NaPi-2) [7, 8, 12]. For NaPi-2, site directed mutagenesis experiments suggested that two major glycosylation sites are used (Fig. 1) [34]. Glycosylation of the transporter might be required for its biosynthesis and membrane delivery but seems not to be crucial for transport activity [34]. Radiation inactivation experiments and electrophoretic separations under reducing and non-reducing conditions suggested that the functional type II

Key words: proximal tubule, endocytosis, P_i-homeostasis, expression cloning, kinetic characterization.

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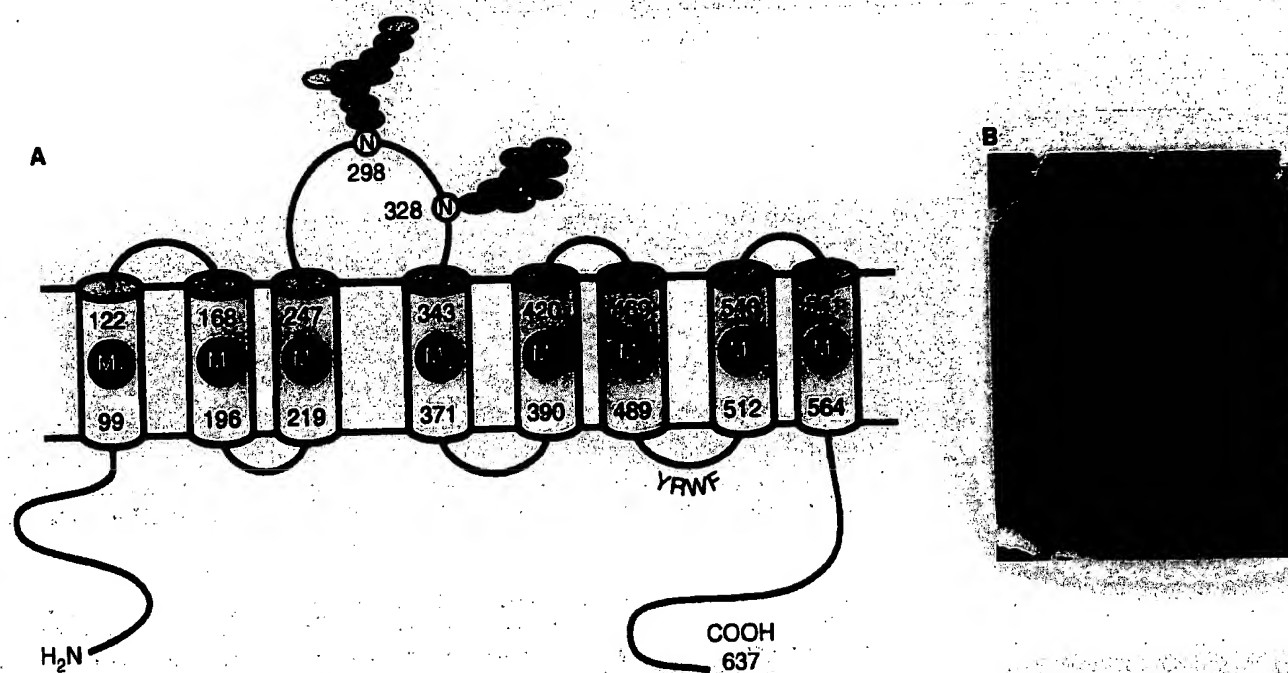


Fig. 1. Structural model for the type II Na/P_i-cotransporter (NaPi-2; rat) based on hydropathy predictions (A). The numbers refer to the amino acid residues. The two glycosylation sites most likely used are indicated (for further description see text). (B) Immunohistochemical localization of the type II Na/P_i-cotransporter (NaPi-2; rat). The brush border location in proximal tubules is evident (for further discussion see text).

Na/P_i-cotransporter could be a homotetramer. Disulphide bonds seem to be involved in forming the multimeric structure, as well as in linking two polypeptides of the 'monomeric' form together that can be cleaved under *in situ* conditions [35, 36].

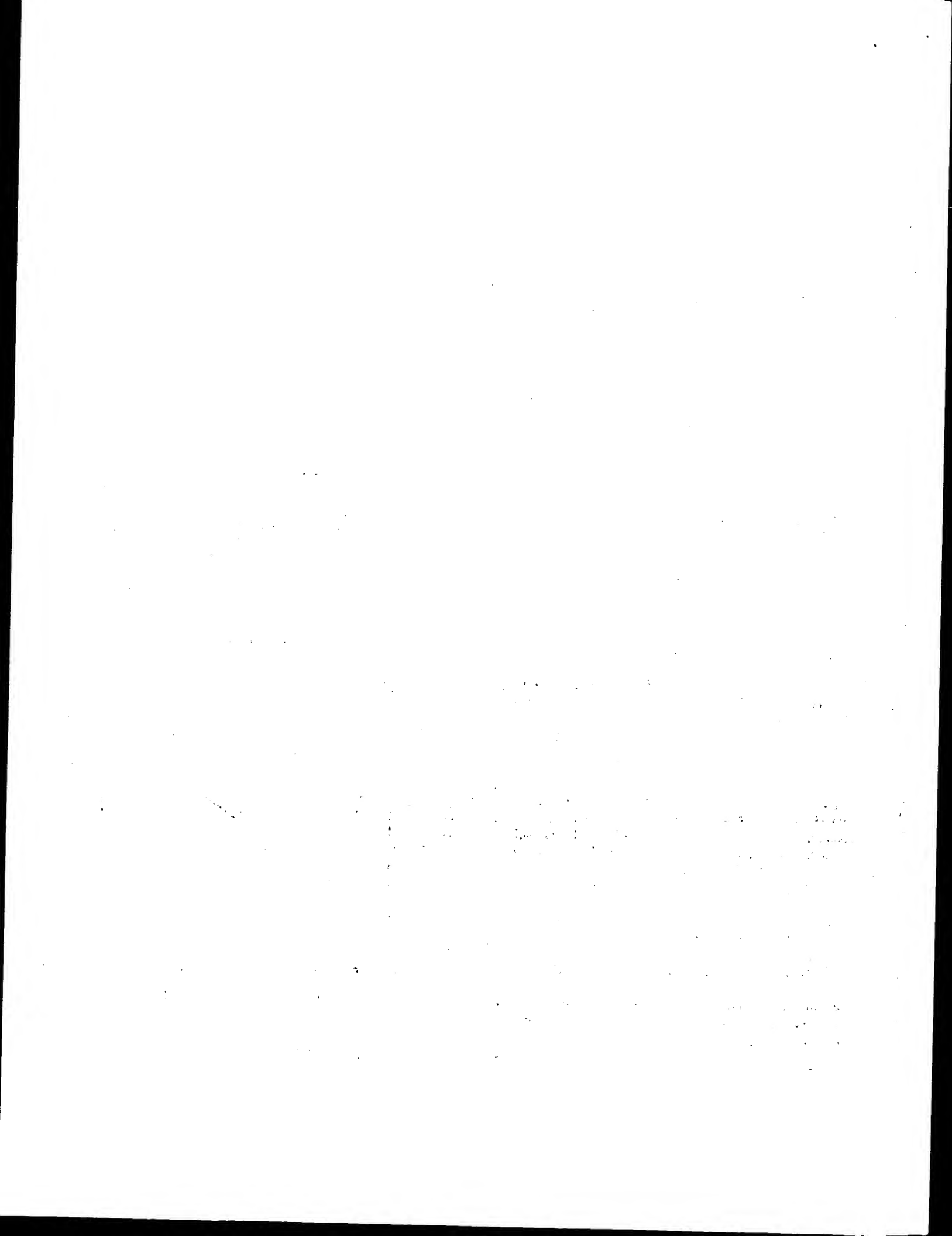
As type I and type II Na/P_i-cotransporters have been identified based on the measurements of sodium dependent P_i-transport into oocytes [11, 12], they fulfill one of the criteria to be P_i-transporters. However, the possibility had to be considered that they may also activate an oocyte 'intrinsic' P_i-transport property. Therefore, additional criteria were required to document their identity as renal brush border membrane Na/P_i-cotransporters. For the type I and type II transporters the 'correct' cellular/intracellular location is good evidence for such functional identity (see above). A detailed comparison of kinetic properties of brush border membrane Na/P_i-cotransport function with those observed after expression of cRNAs in oocytes showed that only the type II Na/P_i-cotransporter meets the required transport characteristics (such as, affinity for sodium and phosphate; respectively, and in particular pH-dependence) [3, 4, 7, 8, 11, 12, 37-39]. Although identified on the basis of its 'P_i-transport function,' it was interesting to find that NaPi-1 (type I) might primarily operate as an anion channel permeable to chloride and a variety of organic anions [39]. Finally, PiT-1 and PiT-2 are not only found in a variety of nonrenal tissues, but their affinity for P_i is in a micromolar range (~20 μM), an affinity not compatible with known brush border P_i-transport function [26, 29].

In addition to its initial transport characterization in tracer studies, the cloned type II Na/P_i-cotransporters (NaPi-5 and NaPi-2) have recently been extensively characterized after expression in oocytes and by measurements of transporter induced steady and presteady state currents (Fig. 2) [38, 40; I. Forster et al.

manuscript in preparation]. Similar to the pioneering studies on the 'cloned' sodium-glucose cotransporter [41, 42], the operation of the type II Na/P_i-cotransporter can be described by a scheme involving transitions between at least six states. We incorporate two Na-binding steps in our model, accounting for the effects of sodium on P_i-affinity as well as on V_{max} of the transporter [40]. Presteady state kinetic measurements indicate that the only voltage dependent steps in the transport cycle arise from the translocation of the unloaded carrier, assumed to be negatively charged, and from the first Na-binding (6 ↔ 1; 1 ↔ 2) [40; and I. Forster, manuscript submitted for publication]. As an extension to the scheme given in Figure 2 we recently obtained evidence for a small slippage of the carrier loaded only with sodium (NaCo in Fig. 2; I. Forster, to be published). At present there is no information on the sequence of events occurring at the internal membrane surface (*trans*-side) because in intact oocytes modifications of internal substrate concentrations are not feasible.

REGULATION OF THE TYPE II Na/P_i-COTRANSPORTER

As discussed above the type II Na/P_i-cotransporter fulfills the characteristics consistent with being the 'main' brush border membrane Na/P_i-cotransporter. This conclusion is further supported by different studies related to the regulation of renal P_i-reabsorption and performed with intact animals and with tissue culture cells (opossum kidney cells; OK-cells; see below) [reviewed in 7-10]. In these studies it was found that different states of altered P_i-transport are associated with altered expression of the type II Na/P_i-cotransporter [23, 24, 43-59]. Given the size limitation of this review we concentrate on two regulatory phenomena: the parathyroid hormone (PTH) and the P_i-deprivation/overload.



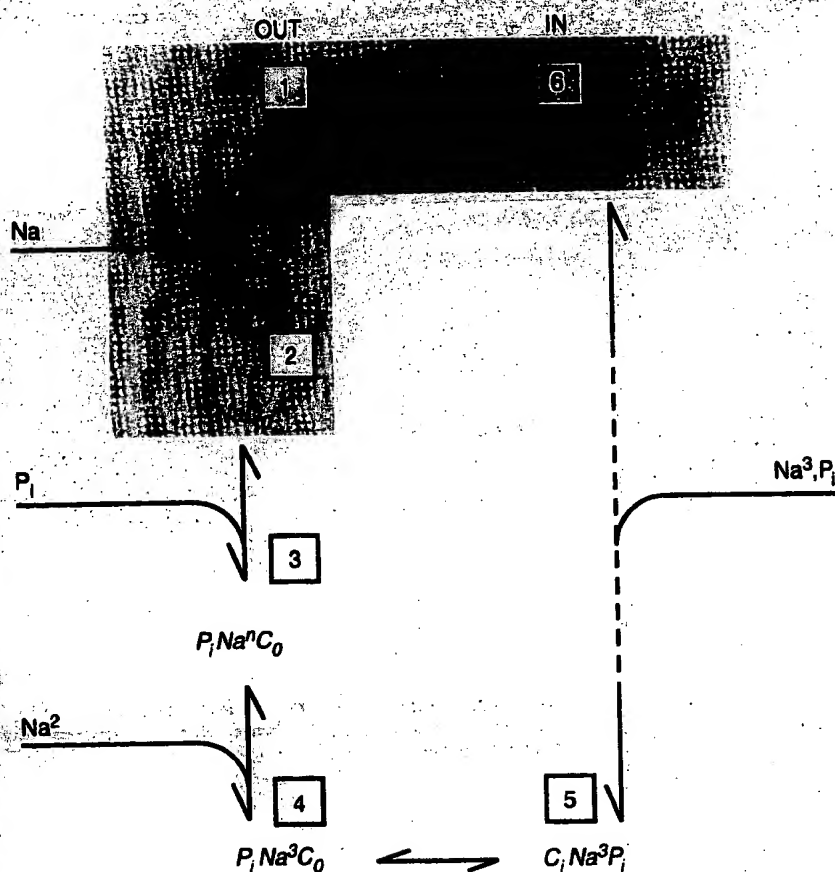


Fig. 2. Kinetic model of type II Na/P_i-cotransporter. The model is based on electrophysiological studies of type II NaPi-cotransporters (NaPi-2 and NaPi-5) expressed in oocytes. Under normal physiological conditions the transporter cycles anticlockwise. A first Na-binding is followed by P_i-interaction and additional interaction with more than one sodium ion. It is assumed that release of sodium and P_i at the inner membrane surface occurs in the reverse mode (mirror symmetry). The shaded transitions (1 ↔ 6, and 1 ↔ 2) represent voltage dependent steps. A Na-leak (slippage) via the partially Na-loaded carrier has recently been identified, but accounts only for a very minor fraction of Na-flux via this transporter (for further discussion see text).

Parathyroid hormone regulation

Parathyroid hormone is a potent inhibitor of proximal tubular Na/P_i-cotransporter. Its effect is associated with a reduction in the V_{max} of brush border membrane Na/P_i-cotransport [reviewed in 1-4]. Transport studies in opossum kidney cells (OK-cells) were very useful for understanding the cellular mechanisms involved in this PTH action. This cell line has preserved properties of proximal tubular P_i-handling, such as expression of an apically located type II Na/P_i-cotransporter (NaPi-4) and its PTH inhibition [19, 60, 61]. Molecular identification of the renal PTH-receptor suggested that PTH action might involve activation of adenylate cyclase and phospholipase C as initial steps [62, 63]. In proximal tubules as in OK-cells, PTH-action can be mimicked by pharmacological activation of the protein kinase A pathway [64, 65; reviewed in 3, 4]. Studies on OK-cells provided evidence that activation of protein kinase C in addition to protein kinase A is also involved in mediating the PTH effect [64-66; reviewed in 3, 4]. It is, however, completely unknown at which intracellular step (such as at the transporter itself) the kinase(s) exert 'their action.' Studies on the action of PTH in OK-cells provided evidence for the involvement of an endocytic step followed by digestion of the Na/P_i-cotransporter [67, 68]. Recovery from PTH inhibition required *de novo* protein synthesis (Fig. 3) [67]. Reducing the endocytosis rate by a high medium osmolarity also reduced the PTH effect on Na/P_i-cotransport [68]. Immunofluorescence studies with rats provided direct evidence for a PTH-induced retrieval of Na/P_i-cotransporters from the brush border membrane, and

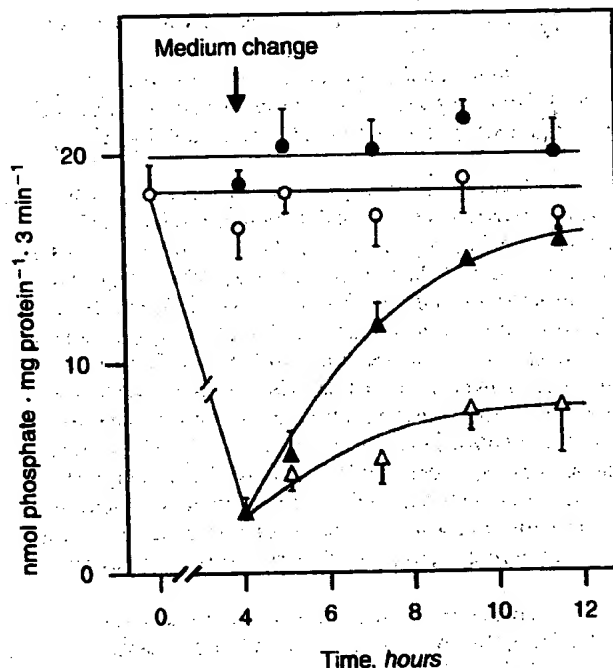
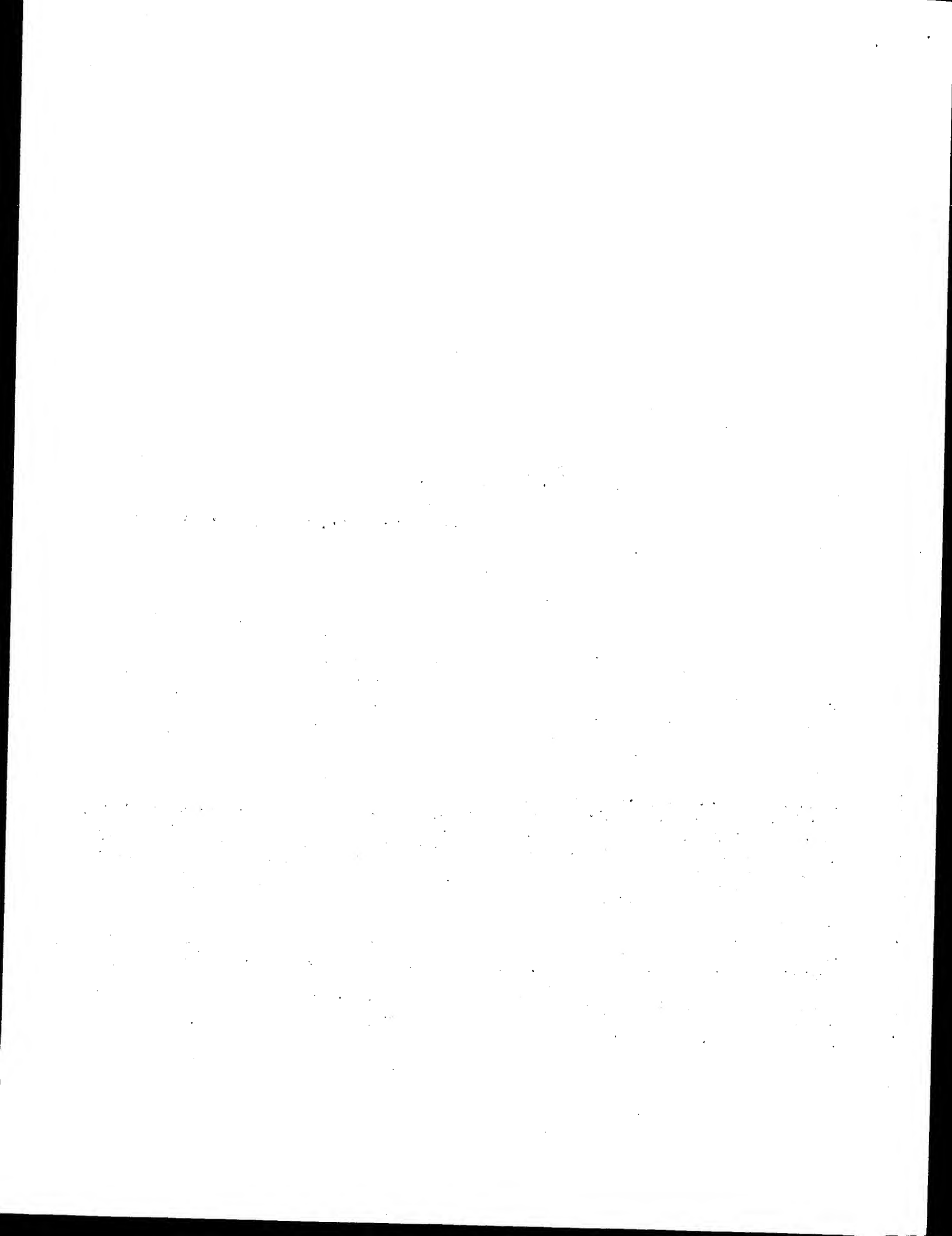


Fig. 3. Evidence for the involvement of internalization/digestion in parathyroid hormone (PTH)-control of the Na/P_i-cotransporter in opossum kidney (OK)-cells. The recovery of transport activity from PTH-inhibition is prevented by blocking protein-synthesis with cycloheximide (for further discussion, see text).



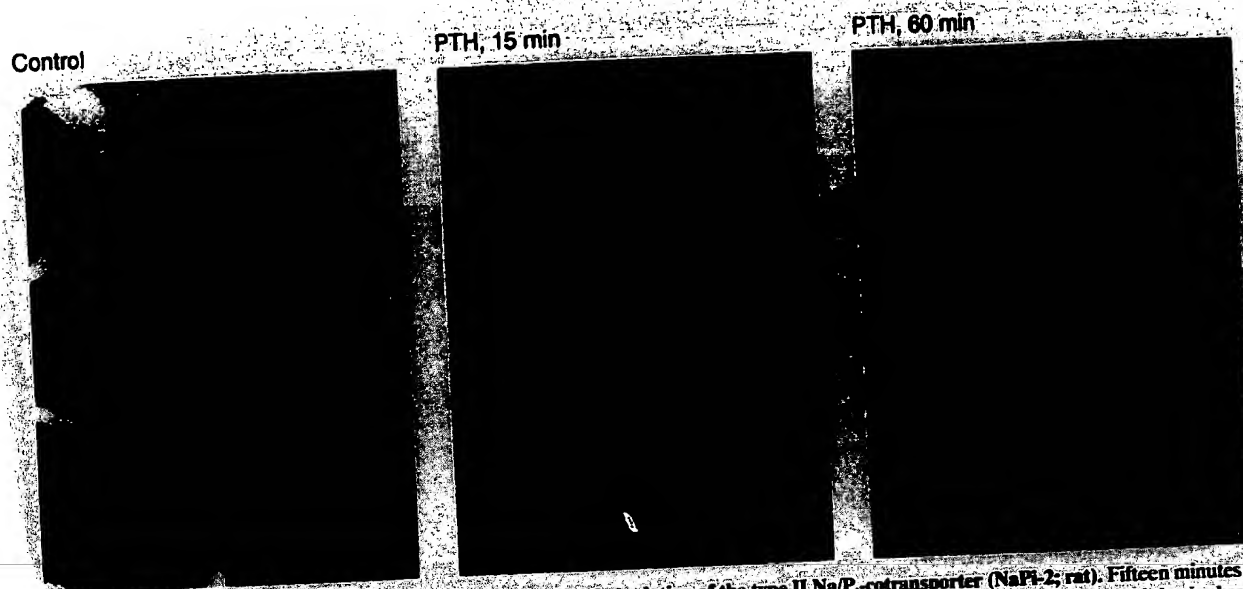


Fig. 4. Parathyroid hormone (PTH)-dependent internalization and degradation of the type II Na/P_i-cotransporter (NaPi-2; rat). Fifteen minutes after injection of PTH, NaPi-2 related immunofluorescence is strongly reduced at the brush border membrane and increased intracellular staining is observed. Sixty minutes after PTH-administration brush border membrane and intracellularly located NaPi-2 related immunofluorescence is further reduced. This experiment suggests internalization followed by degradation (for further discussions see text).

after prolonged time periods also for their digestion (Fig. 4) [45, 69]. More recently, it could be documented in studies on rats that the lysosomal degradation system participates in this PTH-action (J. Biber, J. Keusch and H. Murer, manuscript in preparation). Studies with OK-cells and on the 'intrinsic' (NaPi-4) as well as on a transfected rat type II Na/P_i-cotransporter (NaPi-2) provided the identical information: PTH-induced membrane retrieval and lysosomal degradation of the Na/P_i-cotransporter [57; M. Pfister, J. Biber and H. Murer, unpublished manuscript].

P_i-deprivation/overload

Proximal tubular P_i reabsorption responds to alterations in dietary Pi-intake; these effects are associated with changes in the V_{max} of the brush border membrane Na/P_i-cotransporter [reviewed in 3, 4]. Although this 'adaptive' behavior may involve different hormonal systems in an 'intact' animal (such as PTH), it is suggested that there is an intrinsic cellular mechanism leading to altered brush border membrane Na/P_i-cotransport rate in response to small alteration in P_i-concentrations and/or Ca-concentrations in the plasma and/or tubular fluid [reviewed in 1]. As illustrated in Figure 5, feeding rats chronically (7 days) a high P_i-diet results in a low brush border membrane expression of the type II Na/P_i-cotransporter. On the other hand, chronically feeding (7 days) a low P_i-diet leads to an increased expression. Changes in brush border membrane transporter-expression can be detected already at short-term changes in dietary P_i-intake (2 hour). Although specific mRNA changes are not observed for the acute (2 hour) changes they are observed for the chronic conditions (7 days). Furthermore, it could be shown that the acute (2 hour) response to P_i-deprivation is not blocked by inhibitors of protein synthesis [43, 56, 70; reviewed in 7-20]. The up-regulation is prevented by cholchicine suggesting a microtubule-dependent translocation of presynthesized Na/P_i-cotransporters to the brush border membrane [56]. Recent studies on rats show, in analogy to the PTH effect, that P_i-overload (refeeding) leads to a membrane

retrieval followed by lysosomal degradation [56; J. Biber, J. Keusch and H. Murer, manuscript in preparation]. The 'adaptive' response can also be studied in the OK-cell system documenting the 'intrinsic' nature of this cellular response. This 'adaptive' response cannot be prevented by actinomycin D [71]. Most interestingly recent studies on the 'intrinsic' (NaPi-4) and transfected rat type II Na/P_i-cotransporter (NaPi-2) in OK-cells show a transcription independent increase of the specific transporter protein in response to medium P_i-deprivation (M. Pfister, J. Biber and H. Murer, manuscript in preparation). This strongly suggests that post-transcriptional control mechanisms (protein- and/or mRNA-stability) are crucially involved in this 'adaptive' increase in response to P_i-deprivation [49, 72, 73]. P_i-medium overload in OK-cells leads to internalization and degradation of the transporter, similar to the observation on PTH-inhibition (M. Pfister, J. Biber and H. Murer, manuscript in preparation).

The membrane traffic phenomena involved in PTH- and P_i-dependent control of Na/P_i-cotransport activity are summarized in Figure 6. In both regulatory phenomena the transporter is retrieved from the membrane and can be detected in a 'subapical' compartment. The membrane retrieval mechanism is highly specific as other brush border membrane transport systems are not affected (such as Na/sulphate-cotransporter) [45, 69]. The internalized transporters are finally degraded in the lysosomal compartment. At present there is no evidence that internalized transporters can be reutilized (that is, 'recycled' from a 'subapical' compartment pool). Therefore a 'recovery' from reduced transport activity involves *de novo* synthesis of transporters, which in the short-term is controlled by post-transcriptional mechanisms (such as in 'acute' P_i-deprivation). It is assumed that the outlined mechanisms in control of expression of the type II Na/P_i-cotransporter at the apical cell surface are similar for different regulatory phenomena involved in the control of renal P_i-handling and not discussed in this review. Future studies mainly on the OK-cell system will permit identification of the molecular domains of the

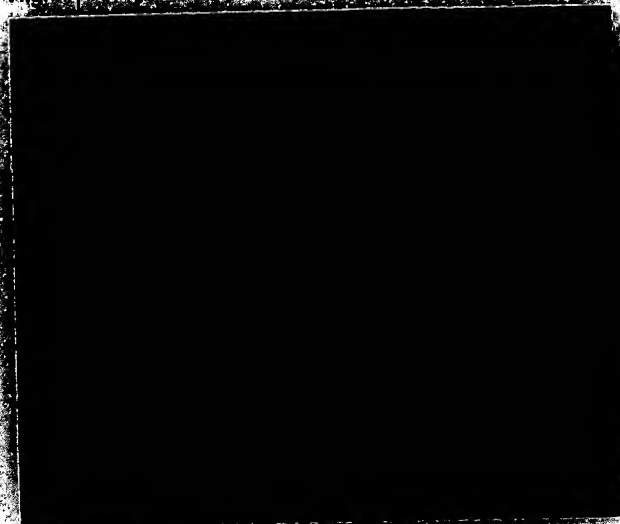
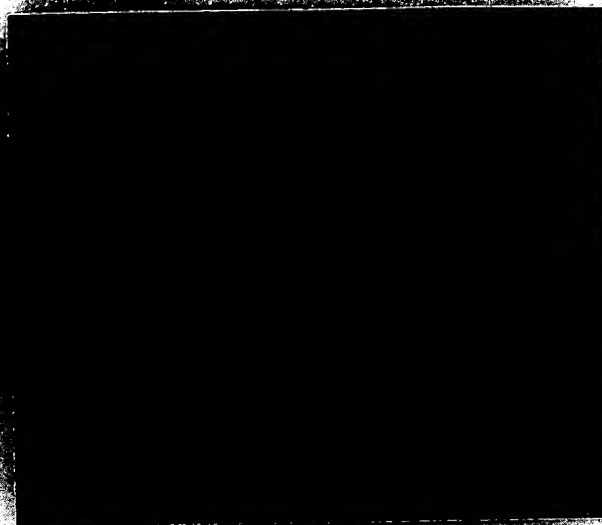
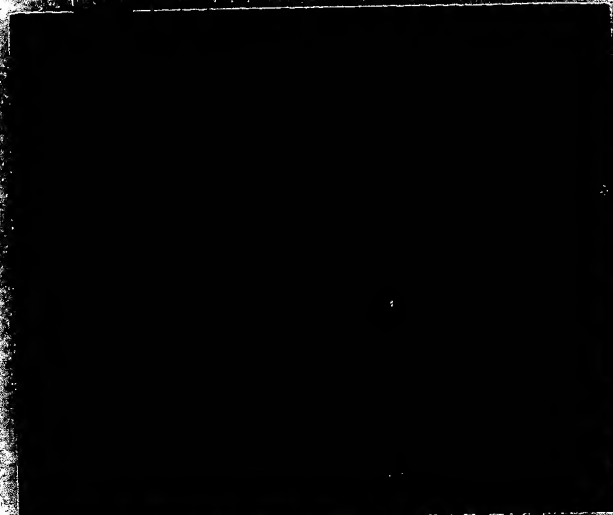
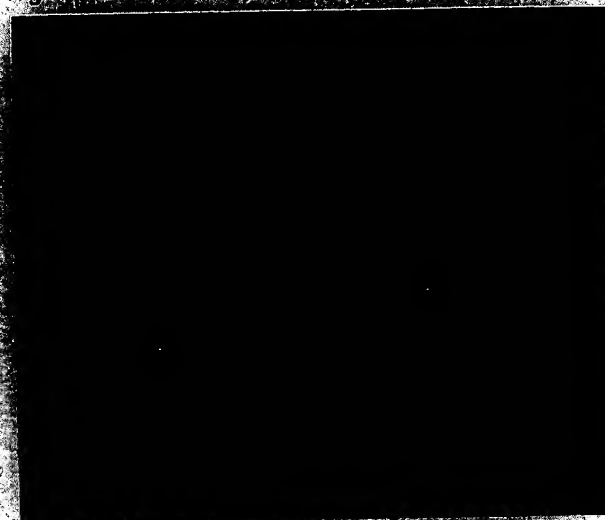
High P_i - chronicLow P_i - 2 hoursLow P_i - chronicHigh P_i - 2 hours

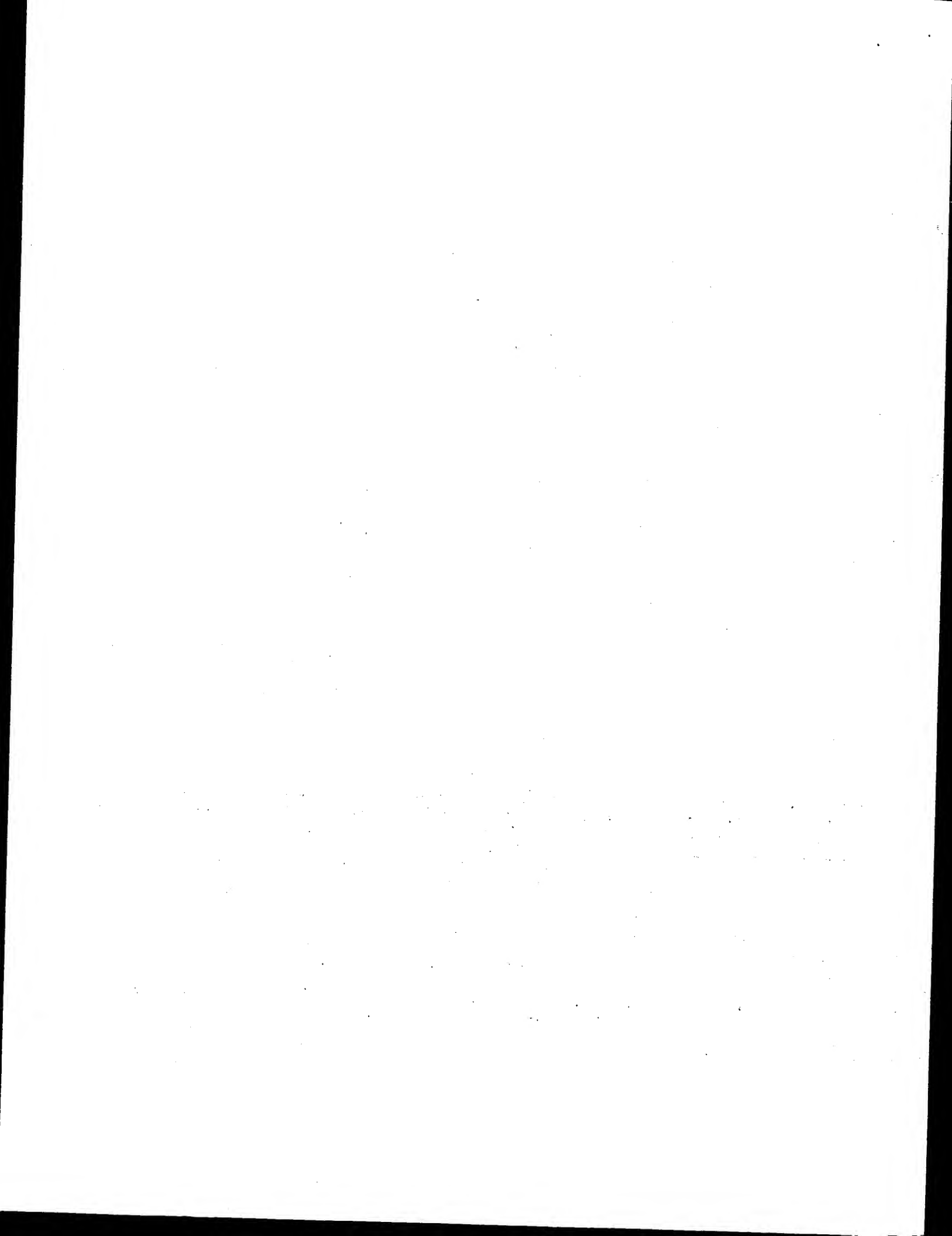
Fig. 5. Dietary P_i induced alterations in brush border expression of the type II Na/P_i-cotransporter. Brush border expression is low for the high P_i-conditions and high for the low P_i-condition. The acute (2 hr) alterations are most likely mediated by protein synthesis independent events, whereas chronic conditions involve protein synthesis or degradation. For further discussion see text.

type II Na/P_i-cotransporter that allow internalization and finally lysosomal degradation to occur. A most important question is also related to the identification/characterization of the intracellular signaling cascade producing at the level of the transporter itself or at the level of an associated regulatory protein the required for internalization/degradation alterations (such as phosphorylation).

GENETIC ALTERATIONS IN PROXIMAL TUBULAR P_i-HANDLING

Different mendelian disorders in P_i-homeostasis have been described and may involve the renal proximal tubular P_i-reabsorption mechanism, that is, the type II Na/P_i-cotransporter [74-76]. These defects are autosomal in nature [for example, hereditary hypophosphatemic rickets with hypercalcuria (HHRH)] or X-

linked, such as represented by the murine *Hyp* and *Gy* homologues of X-linked hypophosphatemia (XLH) [74-76]. The gene structure and chromosomal location of the type II Na/P_i-cotransporter has recently been determined for the murine, human and for the opossum kidney cell isoforms, respectively [77, 78; H. Hilfiker and H. Murer, manuscript in preparation]. The type II Na/P_i-cotransporter has been mapped to human chromosome band 5q35 [79, 80], and the type I Na/P_i-cotransporter to 6p23 → p21.3 [14]. This location excludes a 'direct' role of these genes in the X-linked disorders, but places them as candidates for being affected in autosomal alterations in renal P_i-handling, especially the type II transporter. The gene organizations of the type II Na/P_i-cotransporters in human, mouse and (as far as determined) in opossum kidney cells are highly conserved. In humans and mice



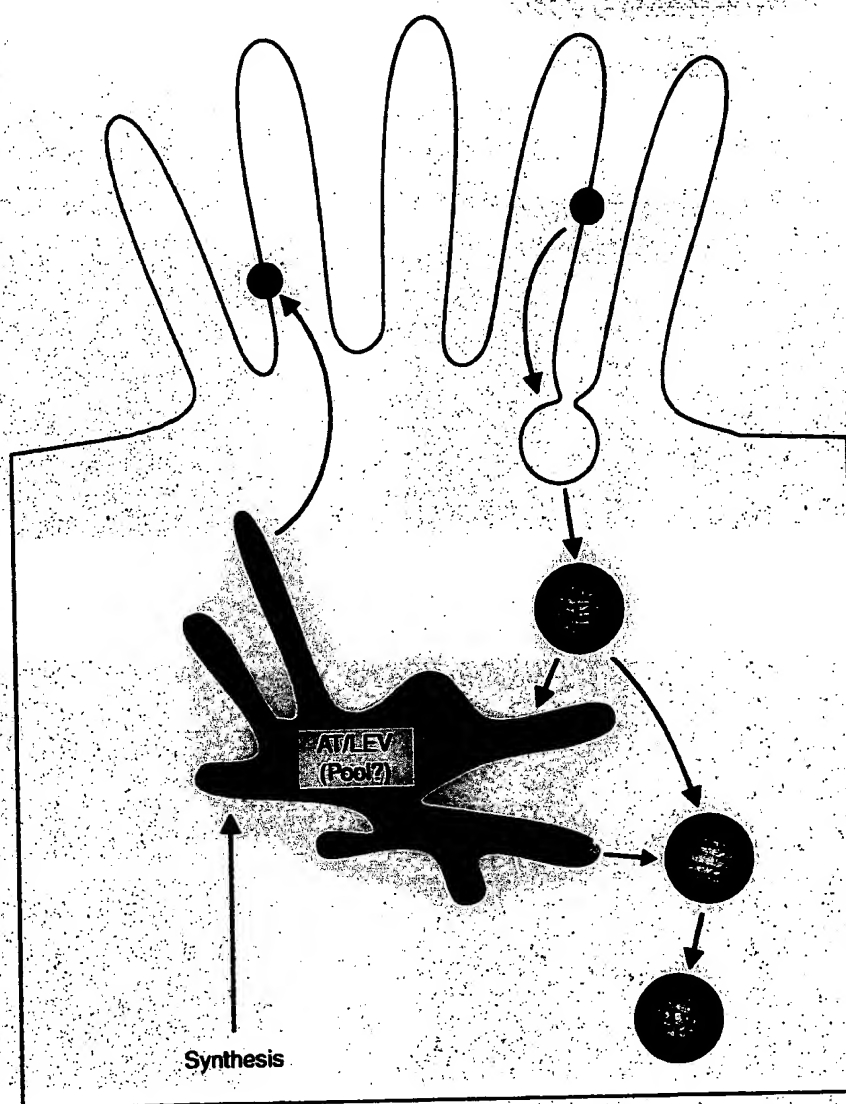
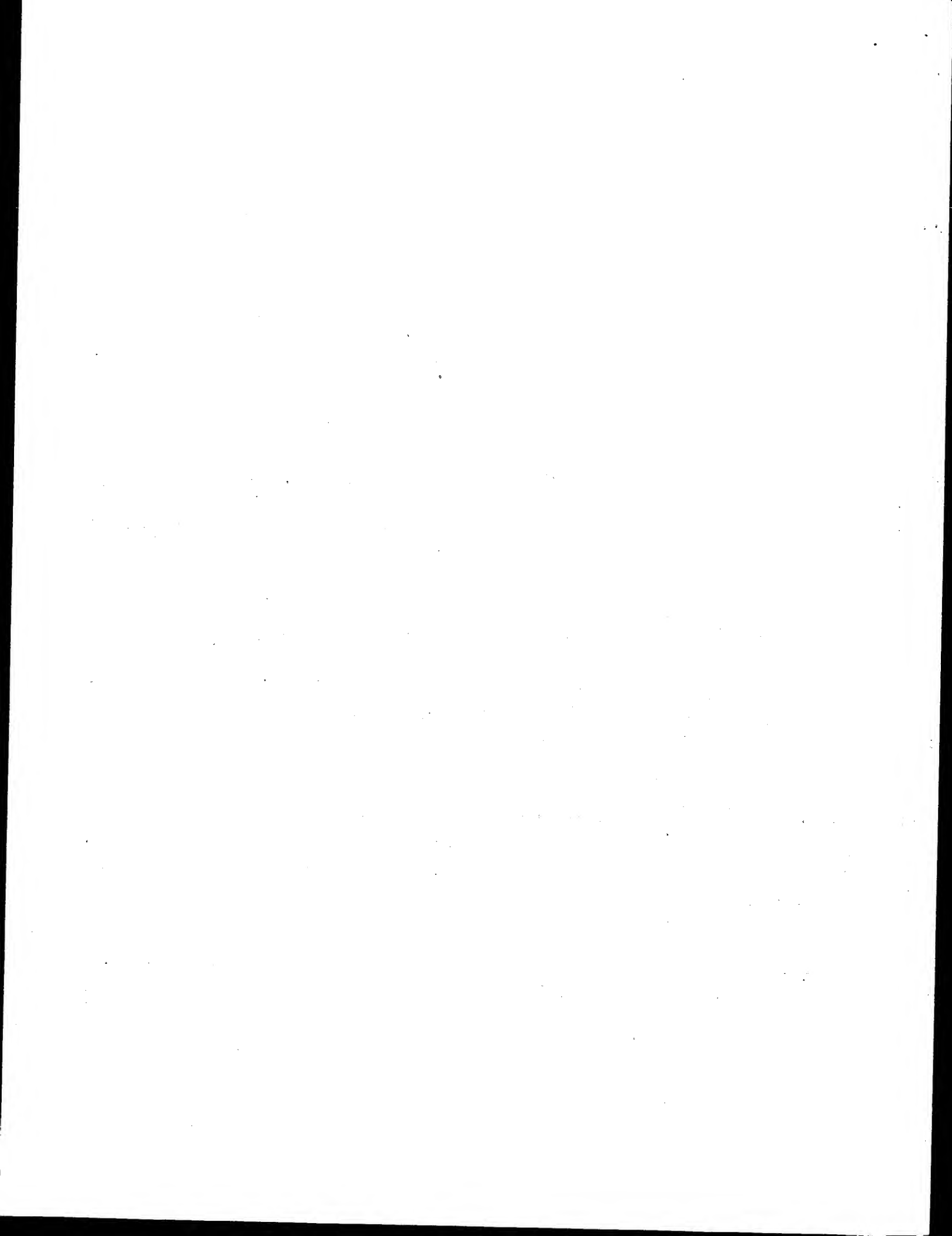


Fig. 6. Membrane traffic events in control of brush border membrane expression of type II Na/P_i cotransporter. For 'down-regulation' transport activity the transporter (small circles) is retrieved from the membrane, appears transiently in a subapical compartment (AT, dense apical tubules; LEV, large endocytic vesicles; SEV, small endocytic vesicles), and finally is degraded in the lysosomes (Ly). For up-regulation the transporter can be inserted 'acutely' into the brush border membrane by a protein synthesis independent mechanism; 'chronic' conditions require *de novo* synthesis. There is at present no evidence for a reutilization of internalized transporters. For further discussion see text.

the genes are approximately 16 kb and are comprised of 13 exons and 12 introns, with intron/exon boundaries located at the 'corresponding' positions of the encoded proteins [77, 78; H. Hilfiker and H. Murer, manuscript in preparation]. Also, the promoter structure has been determined for human, murine and opossum kidney cell type II Na/phosphate cotransporter genes and found to be highly conserved within the first few hundred (~400) bp of 5' flanking sequence, sufficient to drive gene transcription after transfection into OK-cells [77, 78; H. Hilfiker and H. Murer, manuscript in preparation]. Extensive characterization of structure/function relation studies with up to 4.7 kb of 5' flanking promoter sequence of the OK-cell promoter were unable to detect in homologous transfection systems regulatory sequences affecting gene transcription by factors known to be associated with altered activity of the type II Na/P_i cotransporter in OK-cells (H. Hilfiker and H. Murer, unpublished data). These latter data strongly suggest that the identified promoter structures 'only' confine tissue specific expression and that physiological/pathophysiological regulation of this transporter mainly occurs at

the post-transcriptional level. It must be indicated that structure/function relationship studies on the type II Na/P_i cotransporter promoter are made difficult by the fact that, among the different *in vitro* systems available (such as cell lines, primary cultures), only the OK-cell system expresses the type II Na/P_i cotransporter, that is, retains a state of differentiation permitting such experiments.

Recent studies on mice models of X-linked hypophosphatemia have documented that the type II Na/P_i cotransporter, although not located on the X-chromosome, is the 'target' in these diseases and its reduced brush border expression in *Gy* and *Hyp* mice is at the base of the observed renal P_i-wasting [46, 47, 54]. Earlier studies on *Hyp* mice demonstrated that the renal defects in P_i-handling are mediated by a circulating factor [81]. This is in full agreement with the identification of the candidate *PEX* gene [82-84]. The *PEX* gene is in mice preferential expressed in bone cells [82]. The mechanism of action of *PEX* is presently unknown. Based on *PEX*'s proteolytic properties it is suggested that it may be involved in the processing of an endocrine factor involved in the control of expression of the type II Na/P_i cotransporter in the kidney.



CONCLUSION

The type II Na/P_i-cotransporter is a key player in renal P_i-handling, and is therefore also a most important mechanism in overall P_i-homeostasis. The present knowledge of its molecular properties and the application of currently available molecular tools are important to understand the basic properties of this transporter, its physiological/pathophysiological alteration as well as its cell biology.

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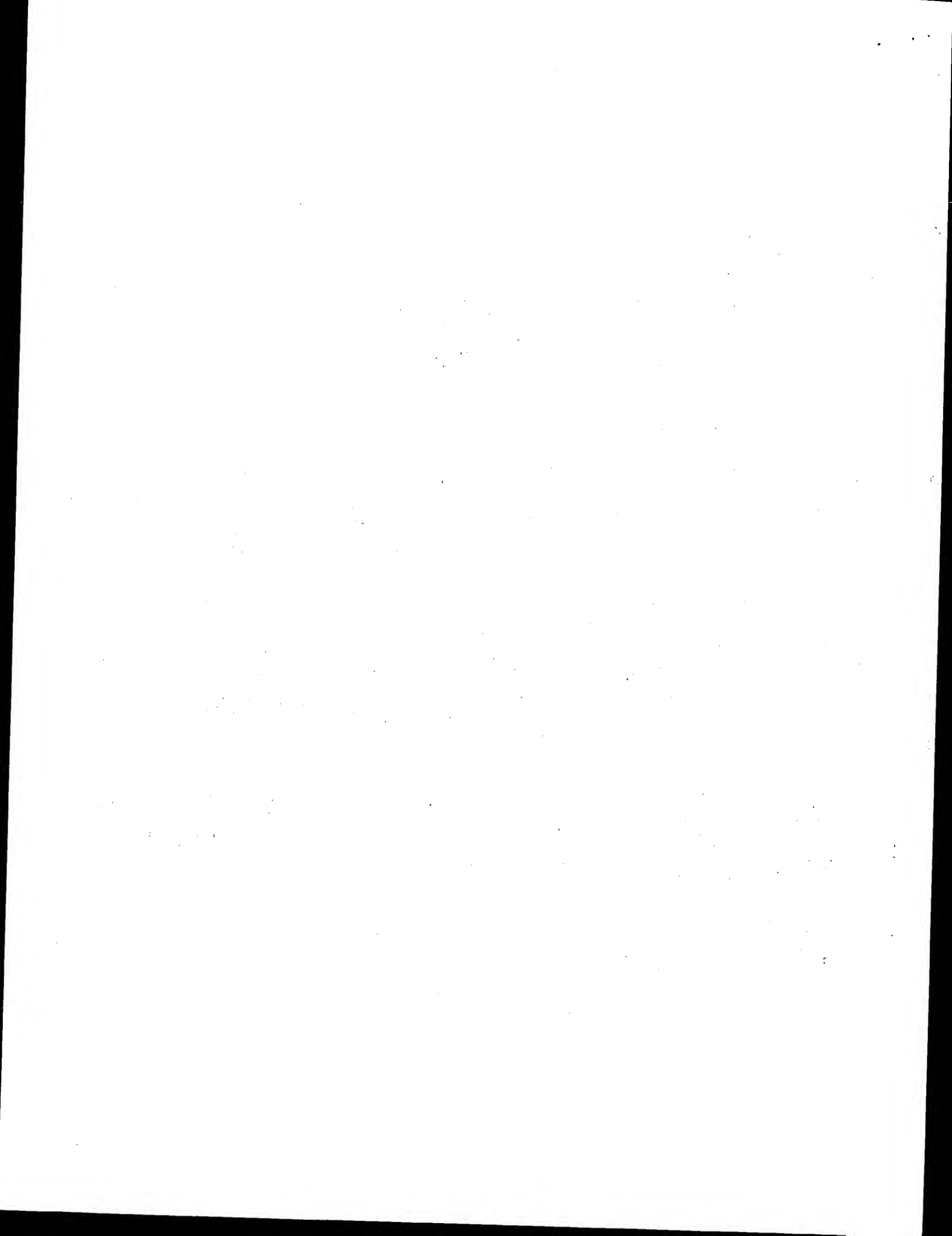
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Strom, T. M. (1); Francis, F.; Lorenz, B. (1); Boeddrich,
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CORPORATE SOURCE: (1) Abt. Paediatrische Genetik, Kinderpoliklinik LMU,
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ACCESSION NUMBER: 1996:355236 BIOSIS
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AUTHOR(S): Strom, Tim M. (1); Francis, F.; Econs, M. J.; Lorenz, B.
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Production of human parathyroid hormone by recombinant *Escherichia coli* TG1 on synthetic medium

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Abstract

Production of human parathyroid hormone (hPTH) by *Escherichia coli* TG1:152cIts was studied. The hPTH is expressed as a fusion protein under control of the bacteriophage λp_R promoter. The organism grows on glucose/mineral salt medium and the expression of the gene product was investigated under variation of temperature and growth rate prior to and after induction. hPTH formation largely depends on cultivation temperature and is optimal for a temperature shift from 30 to 38°C. Product expression is growth coupled and specific hPTH concentration is independent of growth rate. The results are compared with a previous study on *E. coli* N4830:pEX-PPTH grown on complex media.

Key words: *Escherichia coli*; Parathyroid hormone; Thermal induction

1. Nomenclature

F , flow rate of the feed, $l\ h^{-1}$; G , glucose, $g\ l^{-1}$; G_{Feed} , glucose concentration in the feeding solution, $g\ l^{-1}$; hPTH, human parathyroid hormone; K_G , Monod constant for glucose consumption, $g\ l^{-1}$; OD, optical density at 600 nm; pO_2 , oxygen partial pressure in culture medium, % of saturation; t , time after induction, h; X , cell dry mass, $g\ l^{-1}$; $Y_{X/G}$, yield coefficient, $g\ g^{-1}$ (cell dry mass per glucose); V_I , inoculum volume, l; V_L , culture volume, l; μ , specific growth rate, h^{-1} . Index: max, maximum; 0, refers to thermal induction; P, refers to the beginning of product formation.

* Corresponding author.

2. Introduction

Escherichia coli is widely used for the expression of various recombinant proteins. Frequently the cells are cultivated only on small scale on complex media easy to prepare. The media cover known and unknown nutrient requirements of the bacteria. Owing to the low yield coefficient of biomass (usually $< 0.1\ g\ g^{-1}$) high concentrations of complex components are needed to generate sufficient amounts of cells and product (Zabriskie et al., 1987; Harder et al., 1992). Also the downstream processing is more difficult, particularly when an extracellular product has to be separated from the undefined components of the complex medium. In addition, complex components like yeast extract or peptone differ in com-

position and in quality. Therefore, experimental results are often only reproducible if the substrate is taken from the same batch. Many of these problems can be reduced or even avoided using synthetic media. For instance, Riesenberg et al. (1990, 1991) reported on fed batch cultivations with a prototroph *E. coli* strain (TG1) giving high specific production rate and high biomass concentration ($> 100 \text{ g l}^{-1}$) when using a glucose/mineral salt medium and an appropriate feeding strategy. The importance and advantage of using properly defined media in recombinant *E. coli* cultivations have also been emphasized by Fieschko and Ritch (1986).

Different observations have been reported on the influence of the growth rate on protein expression. Zabriskie et al. (1987) studied the production of recombinant malaria antigen. The authors did not find a dependency of product yield on the growth rate used before induction, while Curless et al. (1990) reported a significant influence of the preinduction specific growth rate on the expression level of α consensus interferon. Seo and Bailey (1985, 1986) studied cloned gene product expression (β -lactamase) at different growth rates. In batch cultivations (Seo and Bailey, 1985), wherein the growth medium was adjusted to give different growth rates in the exponential phase, the productivity of recombinant protein decreased steadily with growth rate. In continuous culture (Seo and Bailey, 1986), a qualitatively different behaviour was observed as the gene product activity exhibited a sharp maximum with respect to specific growth rate.

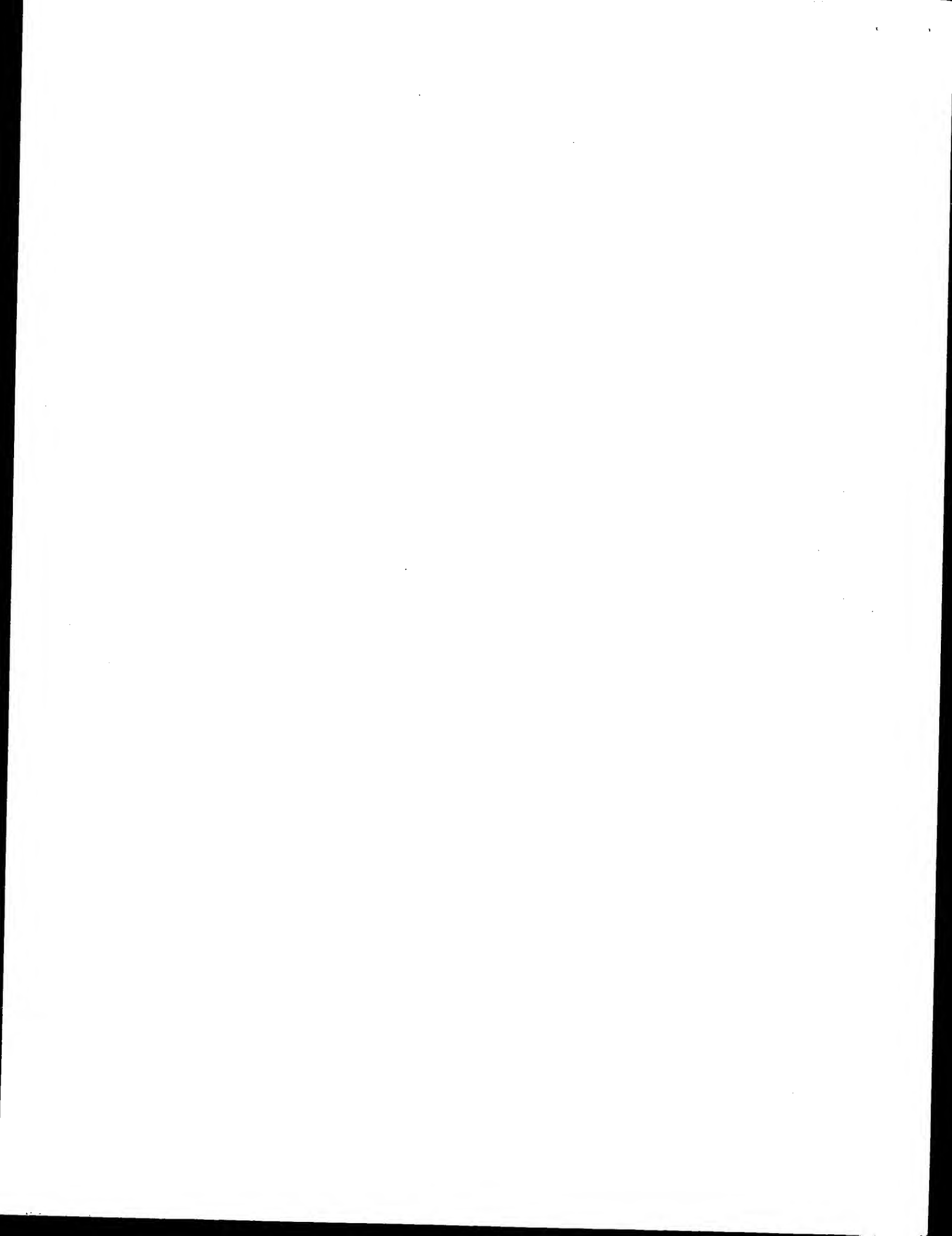
The temperature-sensitive repressor of bacteriophage λ is a well-established device for the control of gene expression. This induction system is easy to handle and has the additional advantage that no more chemicals are required for gene expression. Only a few investigations have been done to find out the optimal thermal shifts for growth and recombinant protein production (Lin et al., 1985; Strandberg and Enfors, 1991; Harder et al., 1992). All these authors suggest that the choice of induction temperature is in the range from 37 to 39°C instead of 42°C as is often recommended (Remault et al., 1981; Sambrook et al., 1989).

In a recent communication, Harder et al. (1992) studied the expression of human parathyroid hormone hPTH(1-84, Pro^{-1}) by *E. coli* N4830: pEX-PPTH. This strain expresses hPTH as a fusion protein under control of the bacteriophage λp_R promoter. High gene product expression rates and product concentrations could be obtained by using complex media and a temperature shift from 30 to 38°C. In the present study *E. coli* TG1 is employed as the host for the I52cIts plasmid (a derivative of pEX-PPTH). *E. coli* TG1 grows up to high biomass concentration, as shown by Riesenberg et al. (1991). It was therefore thought that high hPTH concentration could also be obtained with this strain. Expression of the recombinant protein was investigated under variation of temperature and growth rate prior to and after induction.

3. Materials and methods

3.1. Strain, plasmid and medium

Since direct expression of hPTH delivers only very small amounts a gene fusion was chosen for production (Breyel et al., 1984; Rabbani et al., 1988, 1990; Høgset et al., 1990). The construction of a gene fusion allowed synthesis of a stable hybrid protein to higher concentrations. The gene hPTH was elongated at its 5'-end by a proline codon and fused to a modified *cro-lacZ* gene which encodes the hybrid protein *cro- β -galactosidase* (Stanley and Luzio, 1984). The construction leads to the expression of a protein of 492 amino acids, of which 84 correspond with hPTH (hence about 17%). The hormone can easily be liberated by hydrolysis with formic acid, leaving an additional proline attached to the hormone. The final product is, therefore, hPTH(1-84, Pro^{-1}). The organism used in this study was *E. coli* TG1 (DSM 6056; Carter et al., 1985). The strain was genetically engineered through cloning with the plasmid I52cIts. This plasmid is a derivative of pEX-PPTH (Wingender et al., 1989). Expression is under temperature-sensitive control of the λ phage p_R promoter. In addition to the product gene and the ampicillin-resistance gene,



it carries the information for the repressor protein cI857. All cultivations were carried out on the glucose/mineral salt medium described by Riesenberget al. (1991). Glucose (22.5 g l^{-1}) is the sole carbon and energy source. In addition, the medium contained ampicillin (100 mg l^{-1}) to select transformed cells and glycine betaine (140 mg l^{-1}) as an osmoprotectant (Le Rudulier et al., 1984). These components were added after sterile filtration to the sterilized glucose/mineral salt medium shortly before inoculation. Plates were prepared by adding 20 g l^{-1} agar agar to the medium before it was autoclaved.

3.2. Inoculum preparation and batch operation

The cells were stored in glycerol at -20°C . The suspension was scraped on an agar plate with ampicillin to form a stock culture of single cell colonies at 30°C . A single colony was grown overnight at 30°C . The inoculum volume V_1 for the bioreactor was calculated by $V_1 = V_L (10 \text{ OD})^{-1}$. A 2-l stirred bioreactor (Setric Genie Industriel, France) was used in this study. The working volume was 1.2 l. Temperature was monitored and controlled. The pH was controlled at 6.8 through addition of 25% ammonia. Filter-sterilized air and oxygen were supplied to the medium and the impeller speed was regulated to keep the $p\text{O}_2$ at 50% of saturation. In batch experiments cells were cultivated at the preinduction temperature until the cell density reached an optical density of 1.0. Induction was done by shifting the temperature with a rate of 1°C min^{-1} to the desired value.

3.3. Fed batch cultivations with constant specific growth rates

These cultivations were carried out to study the dependence of the specific growth rate after induction (from 30 to 38°C) on the production of hPTH. Constant specific growth rates $\mu < \mu_{\max}$ (0.1 to 0.6 h^{-1}) could be realized over a certain time period by making use of a special feeding strategy for glucose. Glucose consumption is given by

$$V_L \frac{dG}{dt} = -V_L \mu X \frac{1}{Y_{X/G}} + FG_{\text{Feed}} \quad (1)$$

Since $\mu < \mu_{\max}$ the glucose concentration in the batch was always near zero, hence $dG/dt = 0$. The biomass concentration follows from

$$X = X_0 e^{\mu t} \quad (2)$$

From Eqns. 1 and 2 the time-dependent glucose feeding rate $F(t)$ is calculated as

$$F(t) = \frac{V_L(t) X_0}{G_{\text{Feed}} Y_{X/G}} \mu e^{\mu t} \quad (3)$$

By taking samples of appropriate volume the culture volume V_L could be kept constant at 1.2 l. Glucose concentration in the feeding solution (G_{Feed}) was varied according to the desired specific growth rate between 12.5 and 135 g l^{-1} . The yield coefficient $Y_{X/G}$ was assumed to be 0.5 g g^{-1} . A computer was programmed to control the pump rate according to Eqn. 3. All these cultivations were done with a temperature shift from 30 to 38°C . The $p\text{O}_2$ was controlled at about 50% saturation.

3.4. Cultivations with continuous medium exchange

In this case, the culture medium was pumped continuously through a cross flow filtration module (Enka membrane: Accurel^R 40, filtration area: 0.1 m^2) in recycle mode. The residence time of the cells in the filter loop was about 12 s. Permeate was substituted by fresh medium at a rate of 1.2 l h^{-1} or 2.4 l h^{-1} . The $p\text{O}_2$ was controlled in the bioreactor at about 50% saturation.

3.5. Analytical methods

Growth was followed by measurement of the optical density at 600 nm . Cell dry mass X was determined by drying 1 ml of cell suspension in a vacuum oven at 40°C . Enzymatic test kits (Boehringer Mannheim, Germany) were used for the determination of glucose, ammonia and acetate in supernatants of centrifuged samples. The product hPTH was determined by harvesting, purifying and quantifying the insoluble fusion protein as described elsewhere (Harder et al., 1992). hPTH concentrations were calculated as 17% of the fusion protein.

4. Results and discussion

4.1. Induction temperature

The course of the biomass concentration for batch cultivations is shown in Fig. 1. The temperature has a significant effect on the time at which glucose is consumed and biomass concentration reaches its maximum value. For $T = 36$ and 38°C this is at only 5 h after a raising the temperature from the preinduction cultivation temperature (30°C), while for $T = 42^\circ\text{C}$ nearly 8 h are needed. Also the biomass yields differ, i.e., 11 g l^{-1} for $T = 36$ and 38°C and 9 g l^{-1} for $T = 42^\circ\text{C}$. Acetate is formed as an intermediate metabolite. The maximum acetate concentrations increase with temperature and are in the range 1 to 3 g l^{-1} . According to the work of Luli and Strohl (1990) such concentrations are too low to inhibit growth significantly. It is noticeable that the growth rate is not constant for the higher temperatures applied, particularly at 42°C . Here the growth rate passes a minimum value between 2 and 4 h as can be discerned clearly by evaluating the differential growth rates. Such a growth behavior is difficult to explain. Product generation (in the form of inclusion bodies but given as

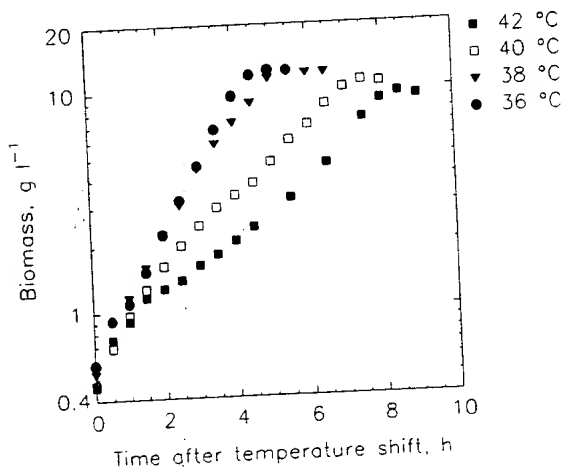


Fig. 1. Growth of *E. coli* TG1:I52clts on glucose/mineral salt medium after induction. The cells were cultivated at 30°C up to an optical density $\text{OD}_{600} = 1.0$. Induction was done by shifting the temperature with a rate of 1°C min^{-1} from 30°C to the desired temperature.

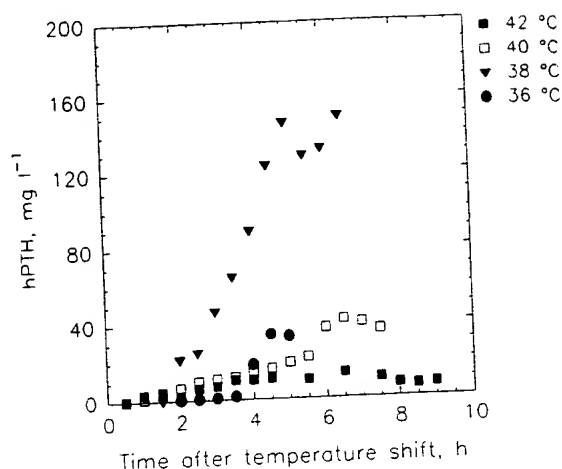
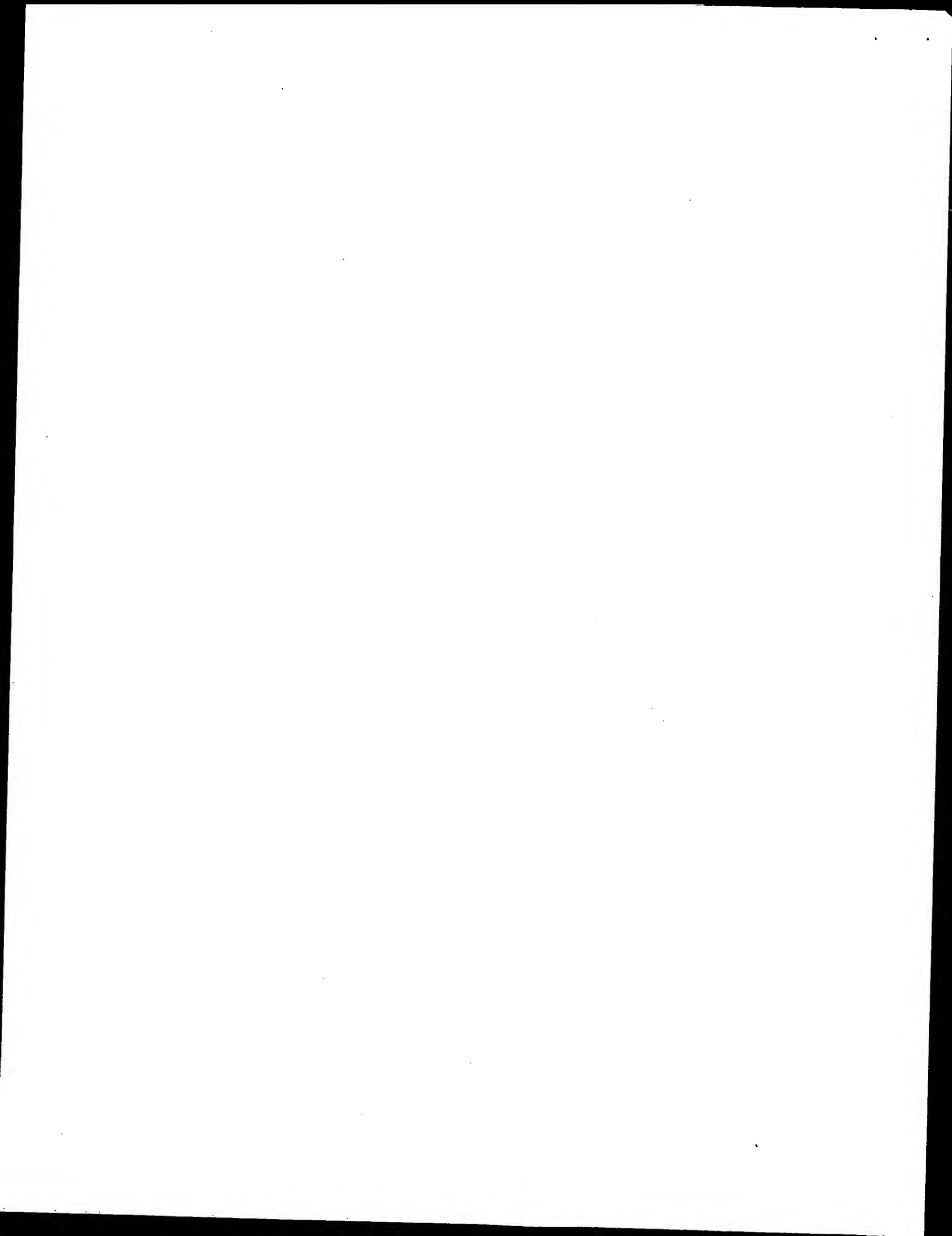


Fig. 2. hPTH formation during growth in batch cultivations. The product (calculated as hPTH) was measured by harvesting an aliquot of the culture suspension, purifying through cell lysis, isolation of the inclusion bodies, gel chromatography and quantifying the fusion protein by densitometry. At 30°C no product could be determined in form of inclusion bodies, hence leakiness of the host/vector system is negligible.

hPTH) is plotted as a function of cultivation time for the four temperatures studied in Fig. 2. *E. coli* TG1:I52clts showed no leakiness during cultivation at 30°C . Product formation does not start immediately after the temperature shift. Obviously, an adaptation phase is needed. More surprising is the sharp temperature dependency. Product formation is low at 42°C but high at 38°C , i.e., roughly 3.5-times larger than at 36 and 40°C . When studying hPTH formation in *E. coli* N4830 grown on complex medium neither an adaptation phase nor a pronounced temperature effect could be observed but similar behaviour to that shown in Fig. 2 was found when a complex medium supplemented with fructose was used (Harder et al., 1992). The findings clearly indicate that medium composition largely affects the thermal response pattern and metabolic cell regulation.

A simple model was formulated to describe biomass formation, glucose consumption and product generation. Owing to the irregularities observed in the growth at higher temperatures (see Fig. 1) the model was applied only to cultivations at 38°C where product formation was largest.



Based on the growth model of Monod biomass and glucose equations are

$$\frac{dX}{dt} = \mu_{\max} \frac{G}{K_G + G} X \quad (4)$$

and

$$\frac{dG}{dt} = -\frac{1}{Y_{X/G}} \mu X \quad (5)$$

Product formation is assumed to be growth coupled after induction by temperature shift and after passing through a lag phase. If X_P is the biomass concentration where product formation starts it follows that:

$$P(\text{hPTH}) = \alpha(X - X_P) \quad (6)$$

for $X > X_P$, assuming $P(\text{hPTH}) = 0$ at $X \leq X_P$. The model specific parameters (μ_{\max} , K_G , $Y_{X/G}$, and α) were determined by using the Nelder-Mead optimization procedure and numerical solution of Eqns. 4-6 with appropriate initial conditions ($X_0 = 0.53 \text{ g l}^{-1}$, $G_0 = 23.6 \text{ g l}^{-1}$) and the experimental value of X_P . Thus, an excellent data fit could be obtained as shown in Fig. 3. The best fit was found with $\mu_{\max} = 0.81 \text{ h}^{-1}$, $K_G = 4.35 \text{ g l}^{-1}$, $Y_{X/G} = 0.48 \text{ g g}^{-1}$, $X_P = 0.79 \text{ g l}^{-1}$, and $\alpha = 13 \text{ mg g}^{-1} (\text{hPTH per biomass})$. The fitted values of μ_{\max} and $Y_{X/G}$ are in the expected range. The high value for K_G is an indication that growth is apparently not only limited by

substrate. The specific product concentration will be discussed later.

4.2. Preinduction temperature

Batch cultivations were also carried out to examine the influence of the preinduction temperature. The range from 24 to 34°C was chosen where gene expression is negligible. After the temperature shift to 38°C an effect of the preinduction cultivation temperature on growth rate and maximum biomass concentration could not be detected. Irrespective of the preinduction temperature the growth rate was $0.7 \pm 0.1 \text{ h}^{-1}$ and the maximum biomass obtained was $11.4 \pm 0.5 \text{ g l}^{-1}$. However, in contrast to these findings a pronounced dependency of the gene product concentration on the preinduction temperature was observed. When increasing the preinduction temperature from 24 to 30°C, the product concentration increases from 87 to 136 mg l^{-1} . A further increase to 34°C in the preinduction phase led to a decline of product concentration (76 mg l^{-1}).

4.3. Fed batch cultivations with constant specific growth rates

Riesenberg et al. (1990, 1991) described a feeding strategy which allows the cultivation of *E. coli* TG1 up to high cell densities at an approximately constant specific growth rate $\mu < \mu_{\max}$. When using linear feeding and an exponentially increasing feeding rate, as outlined in Materials and Methods, biomass concentrations up to 85 g l^{-1} could be reached with *E. coli* TG1 at 30°C. However, when the plasmid I52cIts was inserted in *E. coli* TG1 biomass concentrations of only around 12 g l^{-1} could be obtained. Although glucose was not detectable in the supernatant acetate accumulated up to 20 g l^{-1} . Thus, the growth was strongly inhibited and eventually ceased.

In order to realize constant specific growth rates with *E. coli* TG1:I52cIts the following strategy was employed. Batch runs were carried out at 30°C with a reduced glucose concentration. At biomass concentrations between 0.5 and 2 g l^{-1} the cultivation temperature was shifted to 38°C

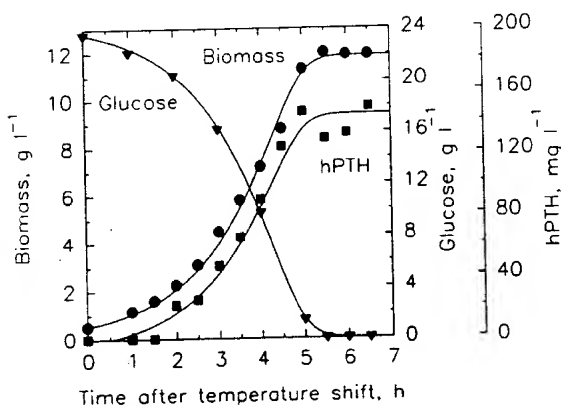


Fig. 3. Time-course of a cultivation with temperature shift from 30 to 38°C. Symbols are experimental data. The lines are calculated by Eqns. 4 to 6.

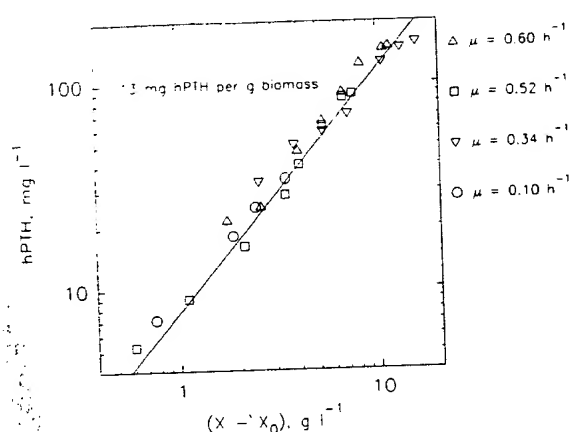


Fig. 4. hPTH formation at different specific growth rates after temperature shift from 30 to 38°C. Specific growth rates were maintained by a special feeding strategy for glucose as given in Eqn. 3.

and glucose feeding was started at a rate given by Eqn. 3 depending on μ . Thus, up to biomass concentrations of around 10 g l⁻¹ constant specific growth rates in the range 0.1 to 0.6 h⁻¹ could be maintained and recombinant product concentration was determined. Fig. 4 shows the hPTH concentration as a function of the biomass concentration for the four different specific growth rates applied. No effect of μ on the product concentration can be observed. There is also no influence of the initial biomass concentration at the time of induction. Hence, one has to

conclude that hPTH expression is independent of the specific growth rate for the particular microbial system employed in this study. The slope of the regression line in Fig. 4 gives a mean specific product concentration of 13 mg hPTH per g biomass and an initial biomass concentration for product formation ($X_p - X_0$) of 0.26 g l⁻¹ which agrees well with the values obtained from batch cultivation (Fig. 3).

4.4. Cultivations with continuous medium exchange

The accumulation of growth-limiting metabolites can be prevented by using a membraned cell recycle reactor with continuous medium exchange (Lee et al., 1989). The flow rates of medium exchange used were 1.2 and 2.4 l h⁻¹. In both cases cell densities of about 40 g l⁻¹ were obtained, in the first case after 4 h and in the latter only after 36 h. At the low flow rate glucose was completely consumed and acetate concentration was less than 1 g l⁻¹. At the higher flow rate glucose and acetate concentration were both over 4 g l⁻¹ and the upper limit of hPTH concentration was reached only 4 h after induction by shifting the cultivation temperature from 30 to 38°C. The time course of this cultivation is shown in Fig. 5. A product concentration of 338 mg hPTH per l could be attained and the specific product concentration was 11 mg hPTH per g biomass.

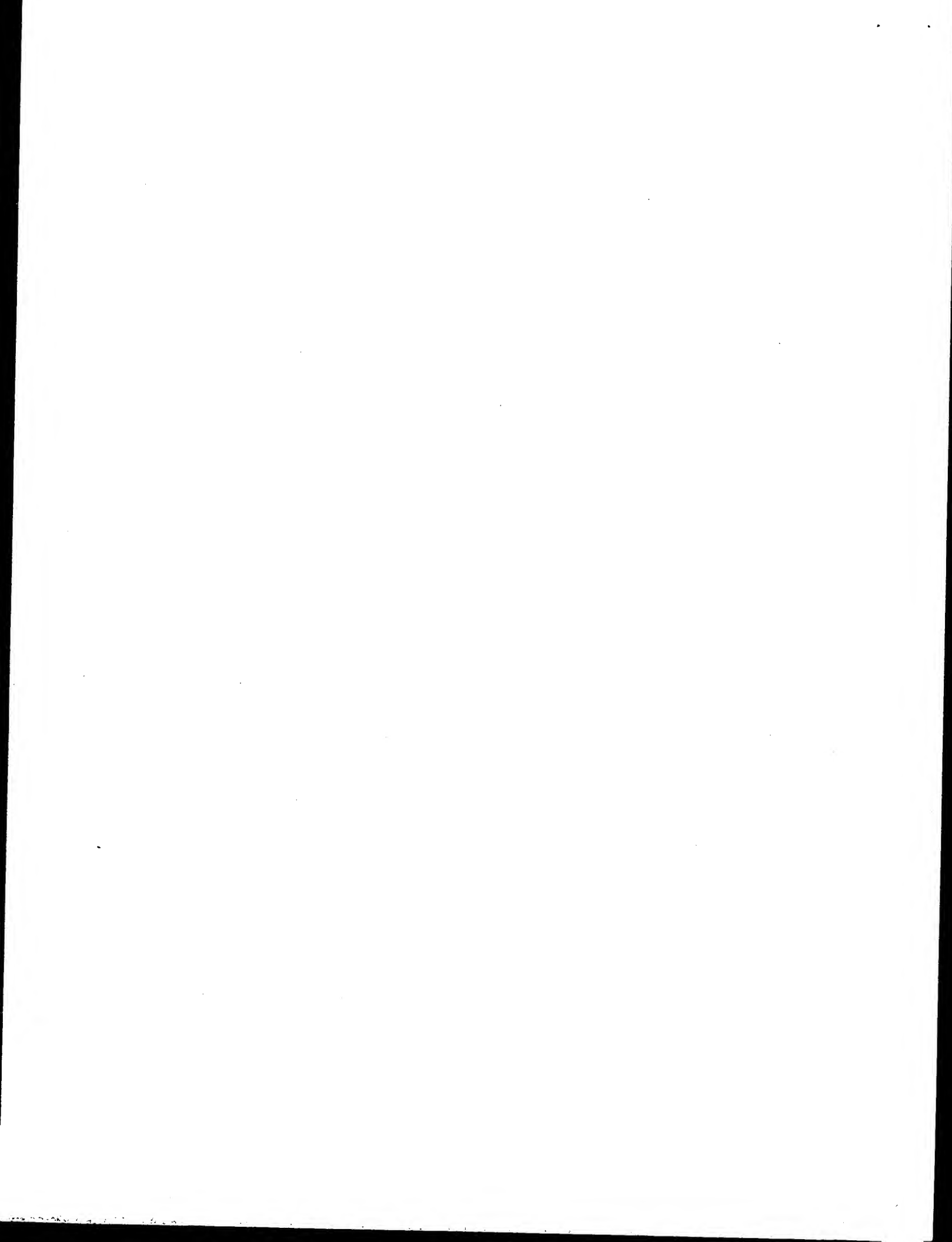
Table 1
Comparison of hPTH expression level in different *E. coli* strains and effect of operation mode

<i>E. coli</i> strain/operation mode	Medium	$X - X_p$ (g l ⁻¹)	hPTH _{max} (mg l ⁻¹)	hPTH _{max} /($X - X_p$) (mg g ⁻¹)	t_{max} (h)	Productivity (mg l ⁻¹ h ⁻¹)
N4830:pEX-PPTH						
Batch	TY4 ^a	4.2	196	46.7	6.5	30.2
Batch	YF ^b	18.3	173	9.5	6.0	28.8
Fed batch	YF ^b	48.8	847	17.4	10.0	84.7
TG1:I52cIts						
Batch/fed batch	synthetic	10.5	136	13.0	5.0	27.2
Cell recycling with continuous medium exchange	synthetic	30.8	338	11.0	4.0	84.5

All results refer to product induction by temperature shift from 30 to 38°C.

^a TY: bactotryptone/yeast extract.

^b YF: yeast extract supplemented with fructose.



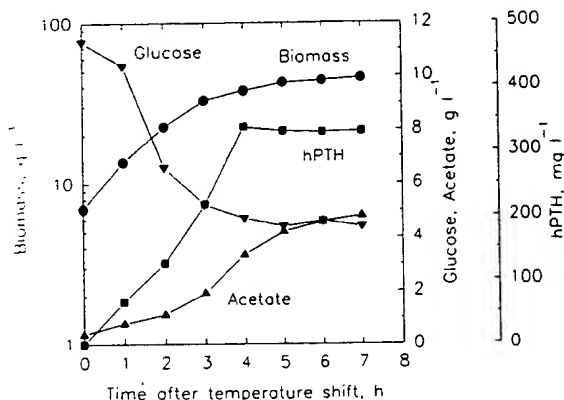


Fig. 5. Time-course of a cultivation with continuous medium exchange after temperature shift from 30 to 38°C. Culture suspension was pumped continuously through a cross flow filtration module in recycle mode. Permeate was substituted by fresh medium at a rate of $2 V_L h^{-1}$.

4.5. Comparison with previous studies

Table 1 summarizes the results of this study with *E. coli* TG1:IS2cIts and the previous findings of Harder et al. (1992) with *E. coli* N4830:pEX-PPTH. The strain N4830 cultivated in batch mode on complex TY medium delivers a low biomass concentration but the highest specific product concentrations. Batch cultivation of N4830 on YF medium gives the higher biomass but a lower specific product concentration. However, the volumetric productivities for both media are comparable and batch as well as fed batch cultivation of TG1 on synthetic medium both exhibit only a slightly reduced productivity. Higher productivities were obtained in the fed batch cultivation of N4830 on YF medium and in the cultivation of TG1 with cell recycle and continuous exchange of synthetic medium. Although gene product concentrations differ considerably in these cultivations, the productivities are identical as a result of differing process times. In summary, it can be concluded from the data of Table 1 that cultivation of TG1 on a cheap synthetic medium gives hPTH concentrations and productivities comparable with those from N4830 growing on complex media.

Productivity
($mg l^{-1} h^{-1}$)

30.2
28.8
84.7

27.2
84.5

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AUTHOR: Carpenter T.O.

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AUTHOR: Strom T.M.; Francis F.; Lorenz B.; Boddreich A.; Econs M.J.;

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Positional cloning of the PEX gene: new insights into the pathophysiology of X-linked hypophosphatemic rickets

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Econs, Michael J., and Fiona Francis. Positional cloning of the PEX gene: new insights into the pathophysiology of X-linked hypophosphatemic rickets. *Am. J. Physiol.* 273 (Renal Physiol. 42): F489–F498, 1997.—X-linked hypophosphatemic rickets (HYP) is the most common form of hereditary renal phosphate wasting. The hallmarks of this disease are isolated renal phosphate wasting with inappropriately normal calcitriol concentrations and a mineralization defect in bone. Studies in the Hyp mouse, one of the murine models of the human disease, suggest that there is an ~50% decrease in both message and protein of NPT-2, the predominant sodium-phosphate cotransporter in the proximal tubule. However, human NPT-2 maps to chromosome 5q35, indicating that it is not the disease gene. Positional cloning studies have led to the identification of a gene, PEX, which is responsible for the disorder. Further studies have led to identification of the murine Pex gene, which is mutated in the murine models of the disorder. These studies, in concert with other studies, have led to improved understanding of the pathophysiology of HYP and a new appreciation for the complexity of normal phosphate homeostasis.

hypophosphatemia; osteomalacia; endopeptidase; X chromosome

THERE ARE SEVERAL hereditary disorders that result in isolated renal phosphate wasting, including X-linked hypophosphatemic rickets (HYP), autosomal dominant hypophosphatemic rickets (ADHR), and hereditary hypophosphatemic rickets with hypercalciuria (HHRH). The genes mutated in these diseases are likely to play important roles in phosphate homeostasis. The existence of multiple forms of hereditary phosphate wasting indicates that control of phosphate homeostasis is a complex process. Understanding the pathogenesis of these disorders will provide insight into this process and may lead to improved therapies for these conditions. This review will focus on recent developments in understanding the pathogenesis of HYP, which is the most common inherited disorder of renal phosphate wasting.

HYP is an X-linked dominant disorder with a prevalence of ~1:20,000. Patients may present with lower extremity deformities, rickets, short stature, bone pain, dental abscesses, enthesopathy, and osteomalacia (22). However, severity of the phenotype varies considerably. Indeed, affected members of the same family may have markedly different phenotypes, and some individuals have only minimal symptoms. Although there is controversy about whether there is a gene dosage effect, recent evidence suggests that the disorder is a classic

dominant condition with equal severity in males and females (89). The hallmark of the disorder is renal phosphate wasting with resulting hypophosphatemia. Patients also have inappropriately normal concentrations of calcitriol (16, 52, 75, 77).

MURINE MODELS

Many insights into the pathogenesis of HYP have been derived from studies of two murine homologs of the human disease, *Hyp* and *Gy* mice. Linkage studies in the mouse have mapped the *Hyp* and *Gy* mutations to a region of the mouse X chromosome syntenic to the human HYP locus (27, 39, 54, 78). The *Hyp* mouse arose as a spontaneous mutation and has been bred on the C57BL/6J background (27). The *Gy* mutation was induced by irradiation, and these mice have been bred on the B6C3H background (54). Both murine models have renal phosphate wasting, impaired mineralization, and growth retardation. However, the *Gy* mouse also has inner ear abnormalities, deafness, hyperactivity, and circling behavior, and the male *Gy* mouse does not survive on the C57BL/6J background (58). Although both mice have been studied, more experiments have been performed with *Hyp* mice. Controversy exists regarding alleged biochemical differences between the two mouse models (see below). Also, in accordance with

the human disease, there are not marked differences between male and female *Hyp* mice (66); however, fewer data are available for *Gy* mice.

PATHOGENESIS OF PHOSPHATE WASTING

Since parathyroid hormone (PTH) is known to prevent reabsorption of phosphate in the kidney, one might expect it to be involved in the pathogenesis of HYP. However, several lines of evidence suggest that this is not the case. First, PTH concentrations are normal in HYP patients (2); second, parathyroidectomy does not alleviate the phosphate wasting in *Hyp* mice (11); and third, Lyles et al. (51) described a HYP patient with concurrent idiopathic hypoparathyroidism who had phosphate wasting once serum calcium levels were corrected. Hence, it is generally believed that PTH is not responsible for hypophosphatemia in HYP, and researchers have pursued other lines of investigation to determine the pathogenesis of phosphate wasting.

In this regard, studies done in the *Hyp* mouse demonstrate that the renal phosphate wasting results from decreased sodium-dependent phosphate transport in the brush-border membrane of the renal proximal tubule (83). Subsequent studies have shown that, although the low-affinity/high-capacity transport mechanism is intact, there is a defect in the high-affinity/low-capacity transporter (81). Indeed, the maximal transport rate (V_{max}) is about one-half of normal with no change in affinity for phosphate, a finding consistent with a decreased transporter number in the *Hyp* mouse. This high-affinity/low-capacity sodium-dependent phosphate cotransporter (*Npt-2*) has recently been cloned (88), and *Hyp* mice have been shown to have an ~50% decrease in *Npt-2* mRNA and protein (9, 84). Studies done in the *Gy* mouse also demonstrate a reduction in V_{max} of the high-affinity/low-capacity transport system (82), and a decrease in *Npt-2* mRNA and protein was also observed by Tenenhouse et al. (80). Conversely, Collins and Ghishan (10) have reported that *Hyp* and *Gy* mice differ in this regard, since they found normal levels of *Npt-2* mRNA in *Gy* mice, with decreased levels of *Npt-2* protein. The localization of human NPT-2 on chromosome 5q35 eliminated it as a candidate gene for HYP (40). However, the data suggest that the HYP gene is involved in regulation of NPT-2 expression.

Despite the above results, it remained unclear whether the phosphate wasting resulted from a primary renal defect or whether it resulted from elaboration of a humoral factor that alters phosphate uptake in the renal proximal tubule. To test the possibility of a humoral abnormality, Meyer et al. (57) performed parabiosis between *Hyp* and normal mice. They found that normal mice joined to *Hyp* mice had a progressive reduction in plasma phosphate over 3 wk and that these mice had a greater renal phosphate excretion index than normal mice joined to other normal mice. Furthermore, after the normal/*Hyp* pairs were separated, plasma phosphate returned to normal levels in the normal mouse within 24 h. At 2 and 7 days these normal mice had "rebound hyperphosphatemia" compared with mice separated from normal/normal pairs

(57). As exciting as these findings were, parabiosis studies have significant limitations. Urine creatinine concentration in normal mice parabiosed to *Hyp* mice was almost twice as high as that in normal mice joined to normal mice. Since urine volume and/or plasma creatinine were not measured, it is difficult to determine whether renal function was altered, although it is likely that these mice simply had more concentrated urine than normal to normal parabiotic pairs. Additionally, some of the fall in plasma phosphate may have been secondary to inanition, since plasma phosphate fell in normal mice joined to normal mice but not as much as the fall in phosphate when normal mice were joined to *Hyp* mice.

To avoid some of the limitations of parabiosis experiments, Nesbitt et al. (61) performed renal cross-transplantation studies in normal and *Hyp* mice. When normal kidneys were transplanted into nephrectomized *Hyp* mice, the kidneys wasted phosphorus. When *Hyp* kidneys were transplanted into nephrectomized normal mice, the kidneys retained phosphorus normally. Thus the defect in the *Hyp* mouse is neither corrected nor transferred by renal cross-transplantation, and the phosphate transport defect in the *Hyp* mouse is not due to an intrinsic renal abnormality. These data have been further confirmed by subsequent tissue culture studies that employed SV40-transformed cells from the S1 segment of the renal proximal tubule from normal and *Hyp* mice. These studies demonstrate that sodium-dependent phosphate transport in S1 proximal tubule cells is not different between cells obtained from *Hyp* and normal mice (62). Similar studies have been performed with SV40-transformed proximal tubular cells from *Gy* and normal mice. These studies also show equivalent phosphate transport between mutant and normal cells (63).

The above data are supported by a case report by Morgan et al. (60), in which a patient with probable sporadic HYP developed renal failure. The patient received a living related renal transplant from an apparently normal sister and redeveloped renal phosphate wasting. Unfortunately, these results are clouded by the observation that ~32% of renal transplant patients have some degree of renal phosphate wasting (68).

Although the above studies established that the defect in *Hyp* mice is not in the kidney, these studies did not determine alternative tissue(s) that contain the defect. Since bone from *Hyp* mice does not mineralize normally, some investigators have focused their efforts on studying osteoblasts from the *Hyp* mouse. An intrinsic osteoblast defect was proposed by Ecarot et al. (18-20) who transplanted periosteal and osteoblasts from normal and *Hyp* mice into the gluteal muscles of normal and *Hyp* mice. As expected, when normal cells were transplanted into *Hyp* mice, mineralization was impaired. However, when *Hyp* cells were transplanted into normal mice, reduction but not normalization of the defect was observed. Although these studies supported the hypothesis that there is a primary osteoblast defect in the *Hyp* mouse, they did not exclude the

possibility that the putative circulating factor in the *Hyp* mouse could have led to an irreversible developmental defect in the osteoblast. Such a situation would be analogous to the permanent developmental defects that are produced in developing neural tissue by lack of sufficient thyroid hormone at a critical stage of development (7).

Further evidence that the *Hyp* mouse elaborates a humoral factor causing the phosphate wasting and that the osteoblast plays a role in the pathogenesis of the *Hyp* defect is provided by the studies of Lajeunesse et al. (43). These investigators found that *Hyp* serum, when added to the culture media for at least 24 h, impaired phosphate transport in primary mouse proximal tubule cultures (MPTC). This inhibition occurred in a dose-dependent fashion. Additionally, conditioned media from *Hyp* osteoblasts, but not normal osteoblasts, inhibited phosphate transport in the MPTC when the incubation period was at least 24 h. These data suggest that the *Hyp* osteoblast is responsible for the release and/or modification of a humoral factor(s) that inhibits phosphate reabsorption (43). It should be noted, however, that these data do not exclude other tissues as important contributors to the pathogenesis of the phosphate wasting and other phenotypic features of the disorder.

The existence of a humoral factor that can impair renal proximal tubular phosphate transport is supported by the existence of tumor-induced osteomalacia. These tumors, which are frequently of mesenchymal origin, result in isolated renal phosphate wasting and inappropriately low serum calcitriol concentrations, both of which resolve when the tumor is removed. Unfortunately, this factor(s), which we have referred to as "phosphatonin," has only been partially purified (6, 23). Since tumors frequently secrete, in abnormal amounts and in an unregulated fashion, substances that have a role in normal physiology, it is plausible that the phosphate wasting observed in patients who have tumor-induced osteomalacia is due to overproduction of a factor(s) that normally controls renal phosphate reabsorption. If such a factor exists, then its role in the phosphate wasting seen in HYP has yet to be determined.

VITAMIN D METABOLISM

In addition to the phosphate-wasting defect seen in HYP, defects in vitamin D metabolism are postulated. Indeed, treatment of HYP patients with phosphate alone does not result in resolution of the osteomalacia (53). A combination of phosphate and high-dose calcitriol is required (31, 33). In the setting of hypophosphatemia, elevated calcitriol levels are anticipated, since hypophosphatemia increases calcitriol concentrations in both animals and humans (34, 37, 65). Several investigators have instead found normal serum calcitriol concentrations in HYP patients (16, 52, 75, 77). Thus HYP patients have a relative insufficiency of calcitriol in light of their hypophosphatemia. Studies in the *Hyp* mouse confirm and expand the observations made in humans (49, 56). Lobaugh and Drezner (49)

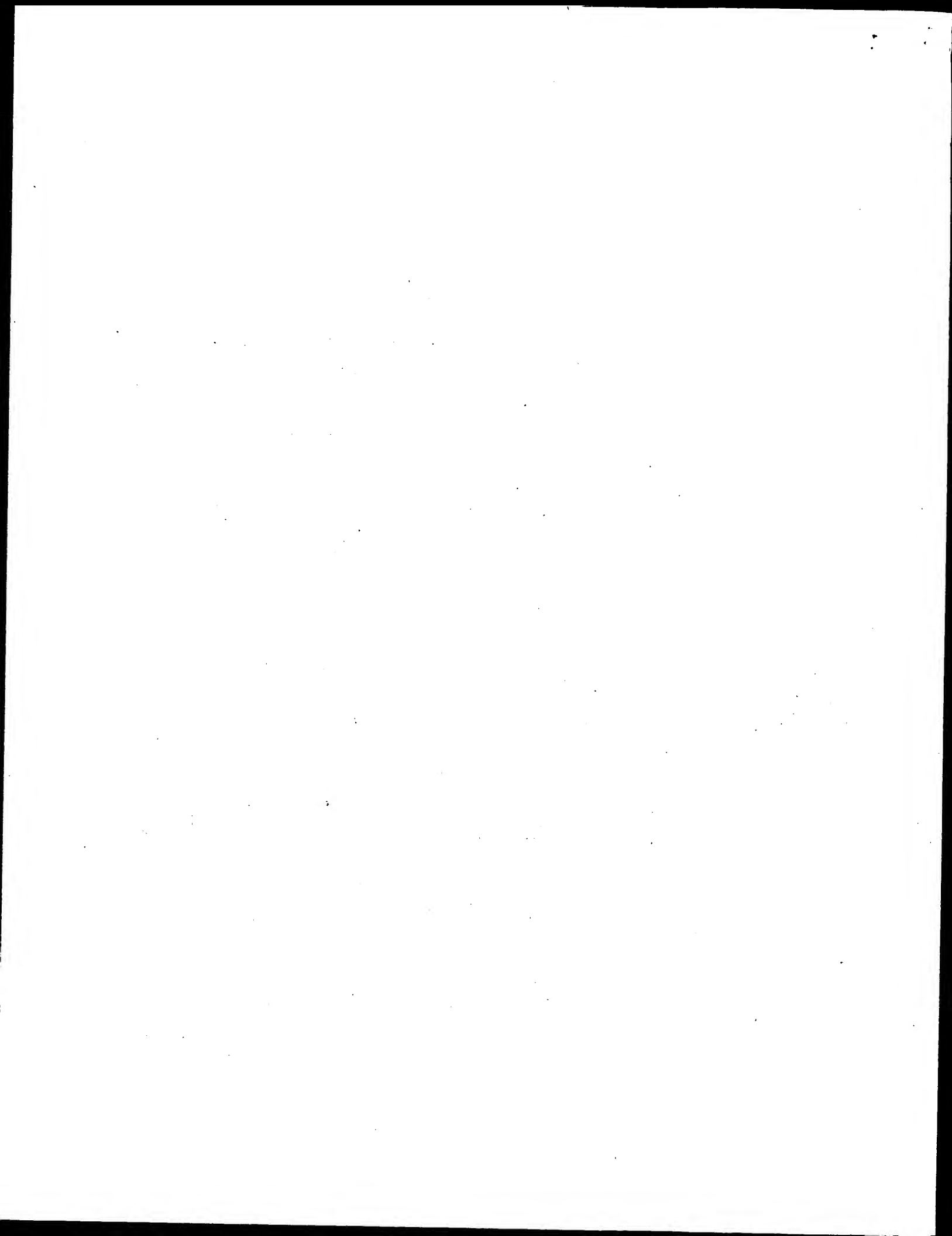
studied the activity of renal 25-hydroxyvitamin D-1 α -hydroxylase [25(OH)D-1 α -hydroxylase] in *Hyp*, normal, and phosphate-depleted mice. This enzyme converts 25(OH)D to 1 α ,25-dihydroxyvitamin D [1,25(OH) $_2$ D], the active form. Although normal mice on phosphate-depleted diets had profoundly increased 25(OH)D-1 α -hydroxylase activity compared with normal mice on the control diet, 25(OH)D-1 α -hydroxylase activity in *Hyp* mice was lower than phosphate-depleted controls, despite similar serum phosphate concentrations. Moreover, 24-hydroxylase is increased in *Hyp* mice compared with controls, indicating that catabolism of calcitriol is probably increased (12, 85). Interestingly, when *Hyp* mice are placed on phosphate-restricted diets, their calcitriol concentrations paradoxically decrease (56).

Studies of vitamin D metabolism in the *Gy* mouse are more controversial. Davidai et al. (14) found that activity of renal 25(OH)D-1 α -hydroxylase in the *Gy* mouse was comparable to that of phosphate-depleted mice and much greater than that of normal controls. Of note, serum calcitriol concentrations were higher in *Gy* mice than controls but not as high as normal mice on phosphate-depleted diets (14). Tenenhouse et al. (82) were unable to detect a statistically significant difference in calcitriol concentrations between *Gy* and normal mice on control diets. Additionally, when *Gy* and normal mice were placed on phosphate-restricted diets, normal mice appropriately increased their calcitriol concentration whereas the calcitriol concentration in *Gy* mice paradoxically dropped substantially below that of *Gy* mice on the control diet. Phosphate depletion also inappropriately increased 24-hydroxylase activity in *Gy* mice, which likely contributed to the paradoxical decrease in calcitriol concentrations. Furthermore, 24-hydroxylase activity was elevated in *Gy* mice on both the control and phosphate-depleted diets, and these investigators concluded that vitamin D metabolism is abnormal in *Gy* mice.

Since interstrain differences could be responsible for the differences in vitamin D metabolism proposed by Davidai et al. (14), Meyer et al. (59) transferred the *Hyp* mutation to the B6C3H background. While studying both mutants on the same genetic background, they did not find a difference in calcitriol concentrations between *Hyp* and *Gy* mice. However, in both *Hyp* and *Gy* mice, calcitriol concentrations were affected by calcium concentrations in the diet. Thus it is possible that much of the observed differences in vitamin D metabolism between *Gy* and *Hyp* mice were secondary to differences in background strain and diet.

POSITIONAL CLONING APPROACH

To gain a better understanding of this disorder, we used the positional cloning approach to locate and clone the HYP gene (38). The advantage of this approach is that knowledge of gene function and tissue expression is not necessary to locate the disease gene. Thus one does not need to make any assumptions about the gene or its function. This approach has been used to identify an ever-expanding number of disease genes (8).



To find a gene using the positional cloning approach, investigators first use linkage analysis to determine the chromosomal location of the disease gene. This method is again used to identify genetic markers that closely flank the disease gene. Once tightly linked flanking markers are determined, a "contig" map is constructed encompassing this region. Contigs are constructed from overlapping pieces of cloned DNA in cosmid, P1, or yeast artificial chromosome (YAC) vectors. Once the region between the flanking markers is covered by the contig, the DNA is analyzed thoroughly to identify all genes present in that region. These genes are tested for mutation in affected individuals.

LINKAGE STUDIES IN X-LINKED HYPOPHOSPHATEMIC RICKETS

Genetic linkage analyses initially localized the HYP gene to distal Xp (55, 67), and further studies by Thakker et al. (86) established the locus order Xpter-DXS43-HYP-DXS41-Xcen. New genetic markers in Xp22 were required to reduce the candidate region to a smaller size suitable for searching for gene transcripts. Examining five large kindreds, we established DXS257 as the closest flanking marker on the telomeric side, and a second new marker, DXS365, was found to be tightly linked to HYP with no recombination events (21). Rowe et al. (73) also found tight linkage with another new marker, DXS274. The linkage analyses until this point had been performed with restriction fragment length polymorphism (i.e., RFLP) markers (4); however, microsatellite markers were being rapidly established as more powerful tools for genetic linkage analysis (48, 87), since they are generally more polymorphic. Through a combination of examining new families and using microsatellite markers generated from the DXS274 and DXS365 markers, recombination events were detected, which led to an ordering of the genetic markers with respect to HYP as follows: Xpter-DXS43-DXS257-DXS365-HYP-DXS274-DXS41-Xcen (24, 26, 71). Hence, DXS365 and DXS274 were the newly established flanking markers on the telomeric and centromeric sides of the gene, respectively. Construction of a YAC contig in this region (described below) later showed these markers to be separated by a physical distance of ~1–1.5 Mb.

CONTIG CONSTRUCTION IN THE CANDIDATE REGION

Xp22 markers (DXS365 and DXS274) were used to screen YAC libraries, constructed from human DNA (1, 44). Three YACs were isolated containing the DXS365 locus, and six were isolated containing DXS274. YACs were subsequently selected for generation of end probes, as well as generation of Alu polymerase chain reaction (Alu-PCR) probes, and these were used to rescreen YAC library filters. An end probe from a DXS274-containing YAC and Alu-PCR products from a DXS365-containing YAC identified several YACs in common. Hence, a YAC contig was constructed consisting minimally of three overlapping nonchimeric YACs as follows: a DXS365-containing YAC, one new YAC (not containing either

DXS365 or DXS274), and a DXS274-containing YAC (29). The total size of the region was estimated to be 1.5 Mb.

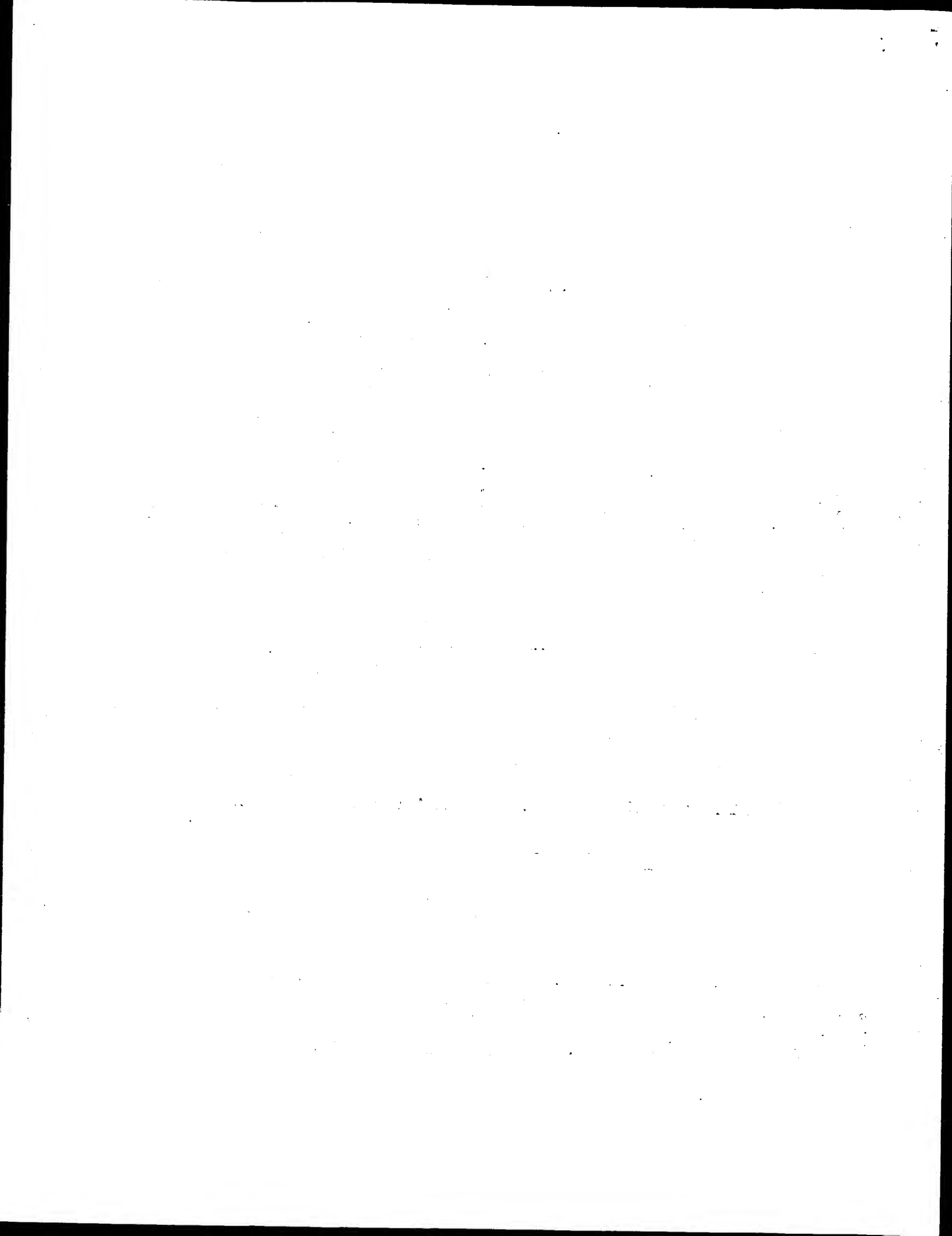
Once the YAC contig was obtained, this was used to isolate new smaller genomic clones [cosmids, P1s, and P1 artificial chromosomes (PACs)] to facilitate the identification of genes in the region. YAC inserts were used to screen gridded cosmid libraries (average insert size 40 kb), a P1 library (average insert size 80 kb), and a PAC library (average insert size 130 kb). These clones were assembled into new contigs by hybridization with end probes, and overlaps were confirmed by restriction digests.

REDUCING THE SIZE OF THE CANDIDATE REGION

Several cosmid and P1 clones were selected for generation of new microsatellite markers. Southern blots containing these clones were screened with a GT-polymer probe to identify fragments likely to contain a polymorphic CA repeat. Sequences were obtained flanking the CA repeat, and primers were designed to amplify the repeat in HYP families. Two new markers were obtained, DXS1683 (25) and DXS7474 (69), and these were examined in 20 large HYP kindreds. Through this analysis, it was possible to place DXS7474 telomeric of HYP (70) and DXS1683 on the centromeric side (26), and these became the closest flanking markers to the disease. Hence, the candidate region could be narrowed to ~350 kb, which is contained within one of the original YAC clones.

ISOLATION OF GENE TRANSCRIPTS IN THE CANDIDATE REGION

Three complementary methods were used for gene transcript isolation, i.e., exon trapping (5), cDNA selection (50), and genomic sequencing. In the exon trapping procedure, genomic DNA (e.g., a cosmid clone) is cloned into a new vector that contains functional 5' and 3' splice sites flanking the cloning site. Upon transfection in mammalian cells, splicing can occur if the cloned genomic fragment contains an exon in the correct orientation. Isolation of RNA and reverse transcription (RT)-PCR experiments allow recovery of the spliced exons. This method was performed for several cosmids and P1 clones in the HYP region. cDNA selection is an alternative strategy involving the hybridization of cDNA libraries to genomic clones, to enrich for cDNAs present in the genomic region of interest. A pool of cosmid, P1, and PAC clones that was known to cover the entire HYP candidate region was prepared. Random-primed cDNA libraries were generated from fetal brain, fetal liver, and adult muscle RNA and hybridized to the pool of genomic clones. Those selected cDNAs were subsequently eluted and cloned. The cDNAs were subsequently arrayed on filter membranes and hybridized systematically with genomic fragments from the candidate region and trapped exons. Positively hybridizing cDNAs were rescreened against the cosmid, P1, and PAC clones to confirm their localization. cDNAs from the entire region were then sequenced and compared with the sequences in the public databases.



To screen for deletions in patients, whole cosmids were hybridized to Southern blots of patient DNAs for the detection of aberrant sized bands or absent bands. In total, ~150 HYP patient DNAs were screened in this way. Four deletions were identified in closely situated cosmids (177, 234, 611, and 1005), implying that the gene causing HYP spanned this region (see Fig. 1). Hence, attention was focused on the DNA contained in these four cosmids; we began genomic sequencing this entire region and closely analyzing the localized cDNAs. In parallel, restriction maps were produced from the cosmids across this region, to verify the extent of the deletions and to more finely map the cDNA clones.

IDENTIFICATION OF THE PEX GENE

The selected cDNA clones had relatively small insert sizes (400–800 bp); however, sequence analysis identified several that were overlapping. In this way it was possible to reconstruct a longer sequence of a gene spanning the patient deletions. Comparison to the sequence databases revealed homologies of this new gene to a family of zinc metalloprotease genes (36), which includes neprilysin (NEP; see Ref. 13), endothelin-converting enzymes (ECE1 and ECE2; see Refs. 28, 74, 90), and the Kell antigen (45). We named this new gene PEX for phosphate-regulating gene with homologies to endopeptidases on the X chromosome (38). By screen-

ing PEX cDNAs against the patient DNA panels, it became obvious that each deletion removed at least one PEX exon. Experiments were then performed to complete the PEX gene structure; sequences of other exon trap products and selected cDNAs were examined for evidence of homology to endopeptidases, RT-PCR experiments were performed to link up these sequences, gene prediction programs were used to recognize exons in the genomic sequence, and a 5' rapid amplification of cDNA ends (RACE) reaction was performed in an attempt to identify the 5' end of the gene. PEX is now known to consist of 22 exons spanning 220 kb of genomic sequence (30), which is the majority of the region defined by the microsatellite markers DXS7474 and DXS1683. Interestingly, the DXS1683 marker is situated in the 3' untranslated region of PEX.

The predicted amino acid sequence of PEX suggests that its protein structure closely resembles that proposed for NEP, ECE1 and ECE2, and Kell, which are type II integral membrane proteins. There is evidence for a short NH₂-terminal cytoplasmic domain, followed by a hydrophobic region, which is likely to be a transmembrane domain, and then a large extracellular domain. This latter domain contains several highly conserved sequence motifs thought to be involved in zinc binding and substrate catalysis. Specifically, these motifs are HEXXH (found in PEX exon 17) and

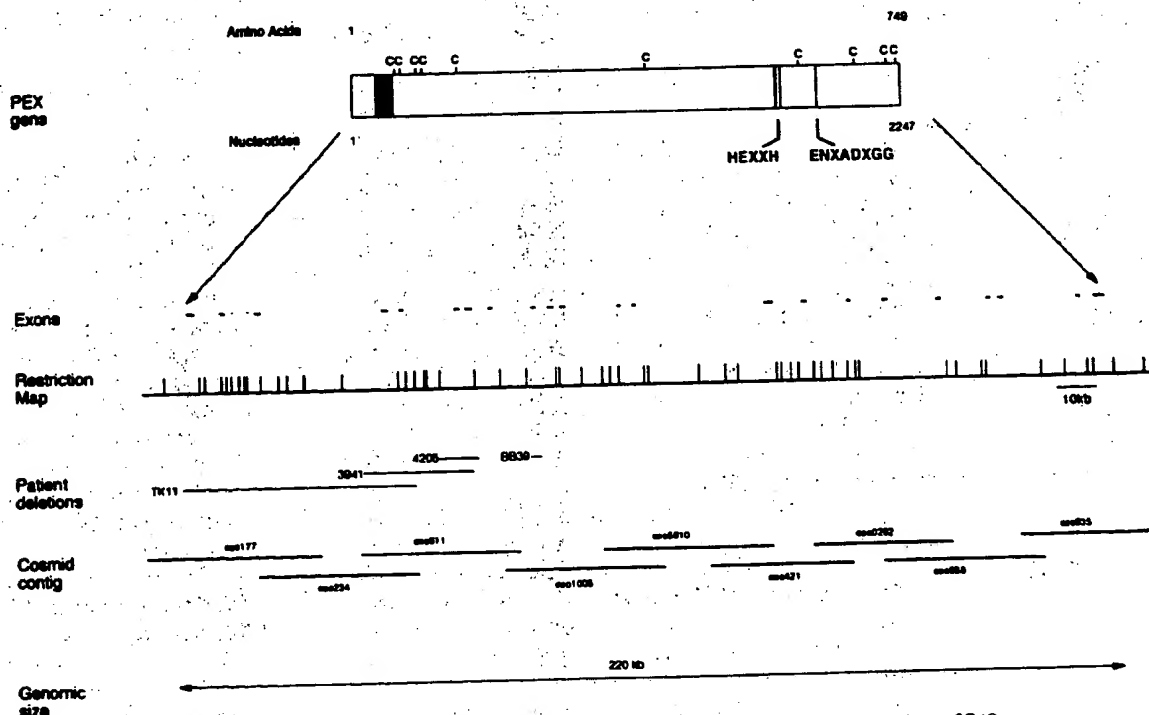
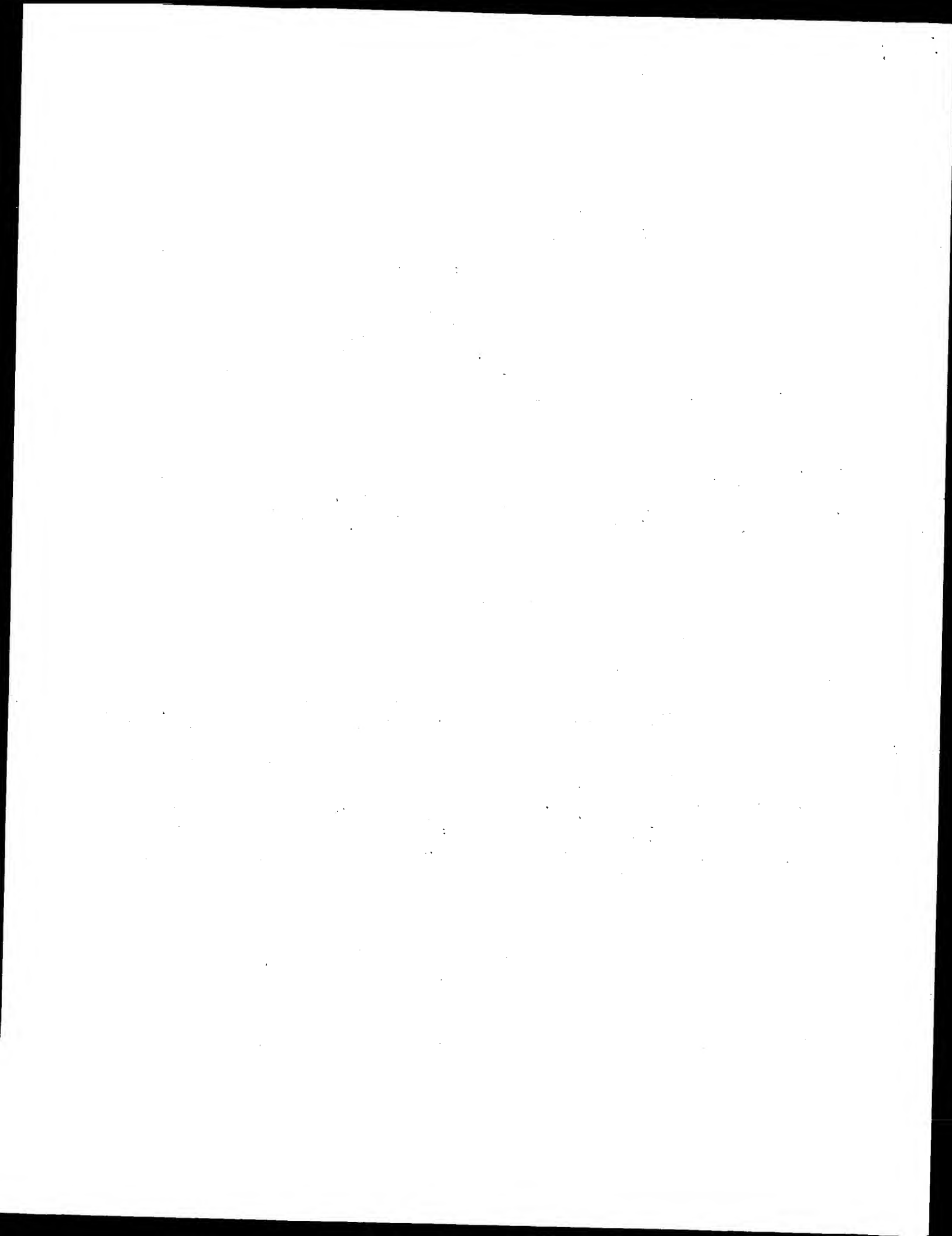


Fig. 1. Schematic representation of the PEX gene. Predicted protein structure of PEX is at top. PEX consists of 749 amino acids and contains hydrophobic sequences likely to be a transmembrane domain (solid box), 10 cysteine residues (C) in the COOH-terminal domain, which are highly conserved in the neutral endopeptidase family, and two zinc binding motifs, HEXXH and ENXADXGG (hatched box and a slim line, respectively). These motifs contain key residues ("H...H" in the first motif and "E" in the second) required for binding a zinc atom. PEX consists of 22 exons, and these are represented below the PEX protein. Several HYP patients have been identified (TK11, 3941, 4205, and 6639) that have deletions of one or more PEX exons (33). A cosmid contig containing 9 overlapping cosmids was constructed to aid the positional cloning of the PEX gene, and genomic sequence analysis has revealed that PEX spans a genomic region of 220 kb.



ENXADXGG (PEX exon 19), in which the underlined residues have been shown in NEP to be zinc binding ligands (46) and the aspartic acid residue has been shown to be involved in substrate catalysis (47). There are in addition 10 cysteine residues throughout the extracellular domain that are likely to be involved in protein folding; these residues are highly conserved in NEP, ECE1 and ECE2, and Kell.

Cloning the human PEX gene has led to a relatively rapid cloning of the mouse Pex cDNA, which has high homology to human PEX (17, 79). Of interest, neither the human nor murine PEX/Pex genes have "classic" Kozak sequences (41). PEX is one of only 3% of known genes that does not have a purine at the -3 position before the ATG initiation sequence (17, 30, 79). This finding may be significant, because, in general, genes that do not have good Kozak sequences tend to be posttranscriptionally regulated (42).

Although little is known about the Kell blood group protein, ECE1, ECE2, and NEP function as ectoenzymes. NEP degrades/inactivates several small peptides including substance P, bradykinin, and enkaphalin. ECE1 and ECE2, on the other hand, convert big endothelin-1 to endothelin-1, the active form (28, 90). Thus it is likely that PEX functions to either activate or degrade a peptide hormone.

EXPRESSION PATTERN

Although RT-PCR can be used to amplify PEX from lymphocyte and fetal brain RNA (38), it is not likely that these are the physiologically relevant tissues of expression. Database searches (17, 30) identified a highly significant match between the 5' end of PEX and an express sequence tag (EST) containing the 5' end of a rat incisor cDNA clone (GenBank accession no. R47026). Thus PEX is probably expressed in teeth and this may result in the high frequency of tooth abscesses in HYP patients. Du et al. (17) detected a 6.6-kb transcript in mouse bone and mouse osteoblasts with Northern blots using a mouse Pex cDNA. In light of the experiments that demonstrate an osteoblast defect in *Hyp* mice, it is not surprising that PEX is expressed in the osteoblast. In a more recent study, Beck et al. (3) detected Pex message from mouse calvaria, long bone, and lung and, to a lesser extent, brain, testis, and muscle by RT-PCR. They did not find Pex message in mouse kidney, heart, or liver. These findings in bone and lung were confirmed by ribonuclease (RNase) protection assay, a method that requires greater expression levels than RT-PCR. Of note, levels of expression of Pex are two orders of magnitude less than that of β -actin (3). They also detected Pex expression on Northern blots of polyA RNA from mouse bone and lung. These investigators also looked for PEX expression in human fetal tissues. They found expression by RT-PCR in calvaria, long bone, lung, ovary, and skeletal muscle (3). RNase protection confirmed expression in calvaria, ovary, lung, and muscle. PEX expression was approximately seven times more abundant in calvaria than in lung, ovary, or muscle (3). Additional data are provided by Grieff et al. (32), who found PEX expression in adult

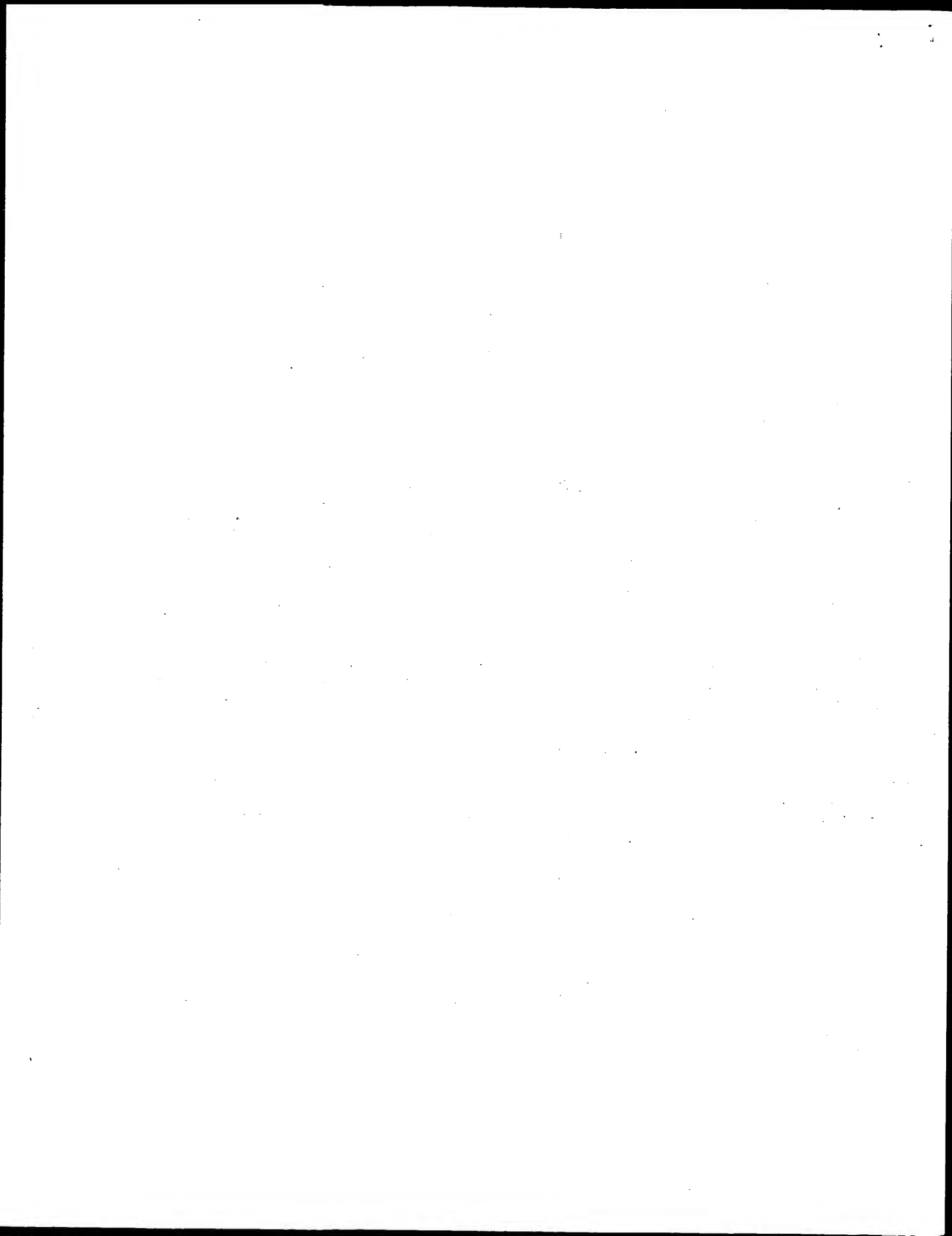
ovary and lung as well as fetal lung and liver. In light of these findings there are several tissues that could play a role in phosphate homeostasis, and PEX may have roles in processes unrelated to phosphate homeostasis.

PEX MUTATIONS

To establish with certainty that PEX is the HYP gene, we looked for point mutations in HYP patients. In our initial efforts, we detected a frame-shift mutation caused by the loss of a TC dinucleotide in PEX exon 6. We also found two point mutations in the splice acceptor site of the PEX exon 7. These two mutations led to exon skipping (38). More recent efforts (30, 72) have demonstrated mutations in almost every exon, and PEX mutations have been found by other investigators (15, 35). Although mutation detection is still ongoing, there does not appear to be a single common mutation that results in the HYP phenotype. Additionally, all of the mutations described so far appear to be loss of function mutations.

The cloning of the mouse Pex cDNA (17, 79) provides an opportunity to interpret previous studies of *Gy* and *Hyp* mice in a new light. Since there are phenotypic differences and an alleged biochemical difference between these mice, several investigators have proposed that there were two closely localized genes that when mutated both resulted in phosphate wasting (14, 58, 76). The occurrence of a single recombination event between *Hyp* and *Gy* (54) further supported this conclusion. Strom et al. (79) have, however, determined that the *Hyp* mouse has a deletion of the last seven Pex exons, and the *Gy* mouse has a deletion at the 5' end of the gene that involves the first 3 exons and an undetermined amount of upstream sequence. Hence, *Hyp* and *Gy* are allelic mutations of Pex. The human PEX gene covers a distance of 220 kb of genomic DNA (30), and it is likely that the mouse Pex gene covers a similar distance. Since the *Hyp* and *Gy* mutations occur at opposite ends of the gene, it is not surprising that a recombination event was detected between the two deletions.

Despite the fact that *Hyp* and *Gy* both have mutations in Pex, there are still phenotypic differences between the two mutants. One enticing possibility is that the location of the mutations affects the phenotype. However, there does not appear to be a strong genotype/phenotype correlation in humans (unpublished observations). It has also been suggested that differences in the biochemical manifestations of the mutations may be related to background strain and dietary differences. Of note, when *Hyp* mice are bred onto the B6C3H background (the background on which the *Gy* mouse is bred) some of the mice exhibit circling behavior (58). However, the *Gy* male does not survive on the C57BL/J6 background (58). An alternative possibility is that one or both of the deletions may involve another gene in the region that influences the overall phenotype. Further work is required to provide an adequate explanation. In any event, the identification of the *Hyp* and *Gy* mutations provides strong evidence



that Pex is the only phosphate-regulating gene on this portion of the X chromosome.

POSSIBLE ROLES FOR PEX IN NORMAL PHOSPHATE HOMEOSTASIS

Despite that fact that mutations in the PEX gene are responsible for X-linked hypophosphatemic rickets, its role in the pathophysiology of HYP is not immediately obvious. Several observations should be taken into account when considering possible mechanisms. First, HYP is an X-linked dominant disorder with little, if any, gene dosage effect. In this regard, it is possible that mutations in the PEX gene result in a dominant negative effect. Alternatively, HYP could be a haploinsufficiency disorder where having one-half the normal amount of PEX gene in females (or null amounts in males) could result in the disease phenotype. This latter possibility is favored, because the TK11 patient deletion (Fig. 1) and the murine *Gy* mutation almost certainly result in lack of message production (38, 79), hence ruling out the possibility of a dominant negative effect. Second, as noted above, the *Hyp* and *Gy* mouse mutations result in decreased levels of *Npt-2*, the high-affinity/low-capacity sodium-dependent phosphate cotransporter. Thus it is likely that PEX serves to directly or indirectly regulate the expression of this transporter. Third, studies demonstrating PEX expression in osteoblasts and those studies that demonstrate an osteoblast defect in the *Hyp* mouse indicate that the osteoblast defect is responsible for at least part of the mineralization abnormalities that are seen in the disease. These studies also indicate that the osteoblast could play an important role in regulating phosphate homeostasis, although there is currently insufficient data to fully support this contention. Fourth, the existence of tumors that secrete phosphatonin, as well as parabiosis data in the *Hyp* mouse, support the notion that the pathophysiology of the disease involves the elaboration of a humoral phosphate-wasting factor. Since PEX mutations, which result in the disease phenotype, are loss-of-function mutations and not activating mutations, it is clear that PEX is not phosphatonin. However, the PEX gene product may play a role in regulating the concentration of phosphatonin.

There are several possible roles for the normal PEX protein in phosphate homeostasis. Since PEX is a member of the neutral endopeptidase family, it is possible that PEX degrades/inactivates phosphatonin. Thus mutations in PEX could interfere with this process and result in excessive concentrations of phosphatonin. However, if this is the case, then one might predict that parabiosis between a *Hyp* and normal mouse would rescue the *Hyp* phenotype (i.e., the normal Pex protein might be expected to degrade excessive phosphatonin from the mutant animal). However, parabiosis did not rescue the *Hyp* phenotype. Instead, normal mice, when parabiosed to *Hyp* mice, started to waste phosphate (57). Although resolution of this apparent discrepancy awaits additional data, it is possible that the kidney is exposed to the high phosphatonin level before the PEX protein has a chance to degrade it.

Alternatively, the normal animal may not adequately upregulate PEX to degrade the excessive amounts of phosphatonin generated by the mutant animal. Another potential mechanism of action that is in keeping with an enzymatic role for PEX is that, under normal circumstances, PEX could function to activate a phosphate-conserving hormone. This possibility has become more plausible in light of recent data that human stanniocalcin stimulates phosphate reabsorption when administered to rats (64). However, this model would also predict that parabiosis of normal mouse to *Hyp* mouse would rescue the *Hyp* phenotype. An additional possibility that fits in with the currently available data is that the PEX gene indirectly functions to inhibit the expression of phosphatonin. Thus mutations of the PEX gene would result in overexpression of phosphatonin and lead to renal phosphate wasting.

CONCLUSION

As is frequently the case when disease genes are identified by the positional cloning approach, identification of the gene does not guarantee immediate understanding of the pathophysiology. However, until the PEX gene was cloned, no one had ever proposed that an enzyme played a role in the disease. Cloning the PEX gene has provided investigators with new opportunities to understand the pathophysiology of the disease and normal phosphate homeostasis. Future research aimed at identifying the PEX substrate, PEX tissue expression, and PEX regulation will undoubtedly add to our understanding of the disease. A more complete understanding of the pathogenesis of HYP and other disorders of phosphate wasting will provide vital clues to understanding normal phosphate homeostasis. The available data indicate that control of phosphate homeostasis is a complex but fascinating process.

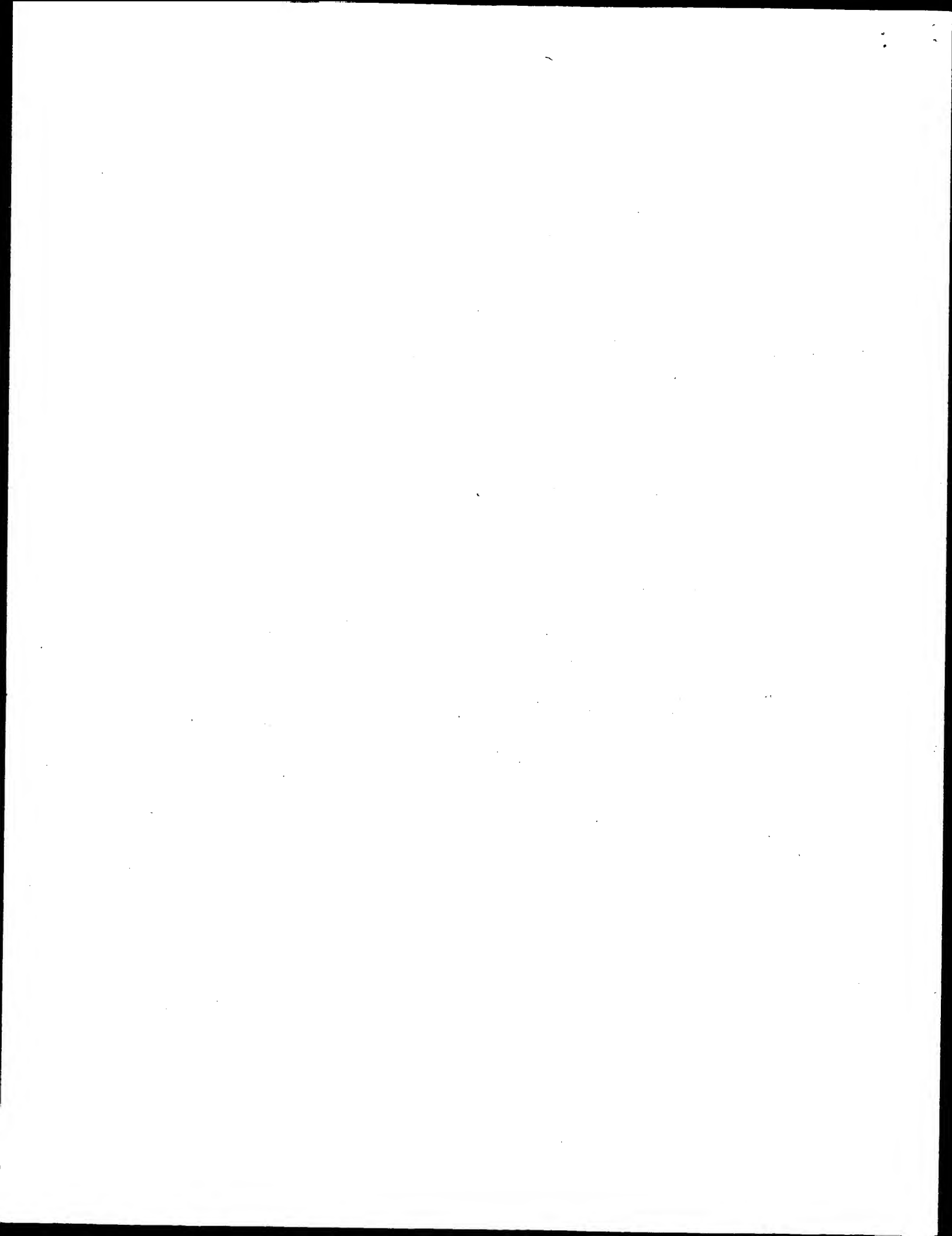
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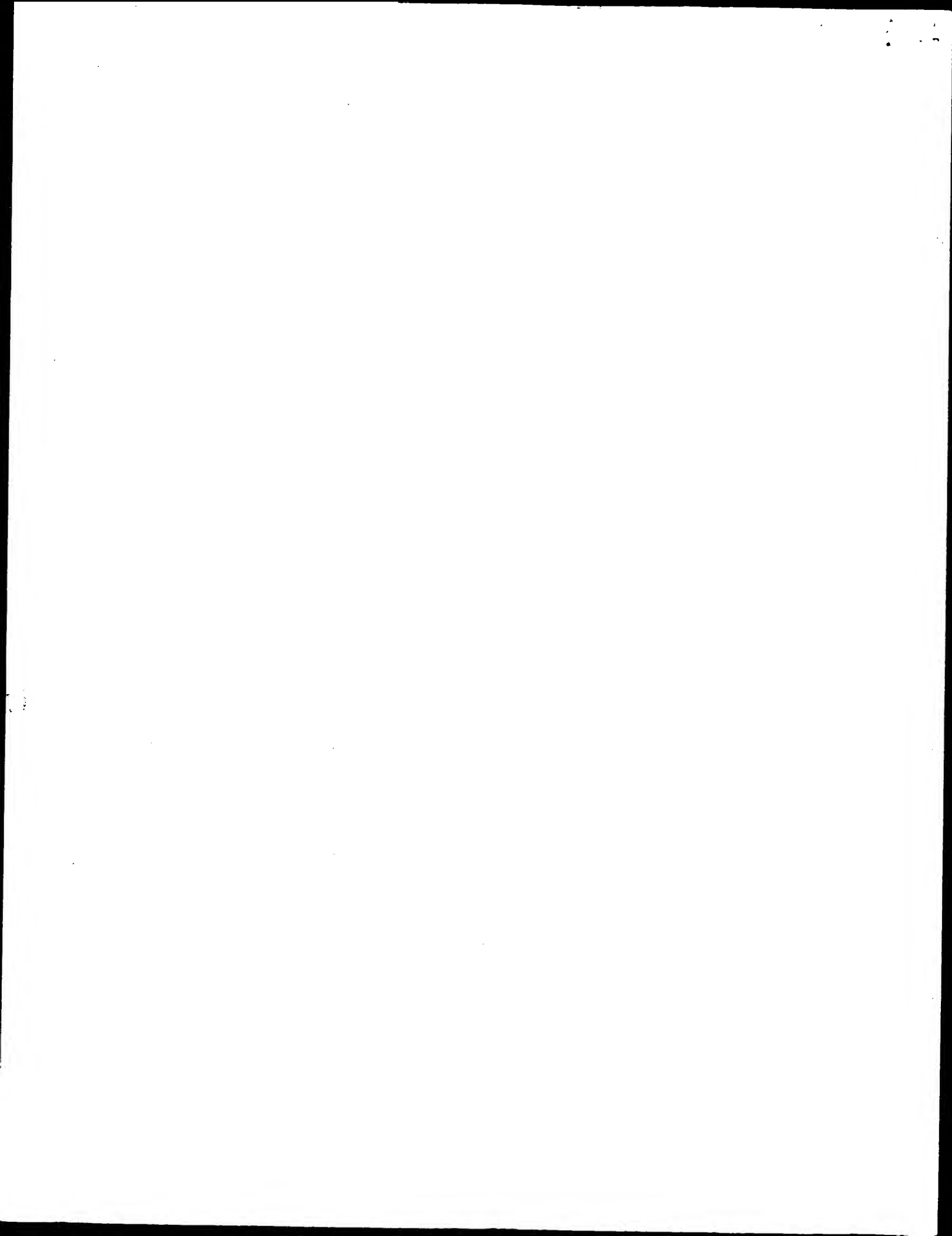
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ACCESSION NUMBER: 1998194311 EMBASE

TITLE: Cloning of human PEX cDNA: Expression, subcellular localization, and endopeptidase activity.

AUTHOR: Lipman M.L.; Panda D.; Bennett H.P.J.; Henderson J.E.; Shane E.; Shen Y.; Goltzman D.; Karaplis A.C.

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ACCESSION NUMBER: 1998385394 EMBASE

TITLE: Genetic screening for X-linked hypophosphatemic mice and ontogenic characterization of the defect in the renal sodium-phosphate transporter.

AUTHOR: Muller Y.L.; Collins J.F.; Ghishan F.K.

CORPORATE SOURCE: Dr. F.K. Ghishan, Department of Pediatrics, Steele Memorial Children's Res. Ctr., Univ. of Arizona Hlth. Sci. Center, 1501 N. Campbell Avenue, Tucson, AZ 85724, United States

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LANGUAGE: English

SUMMARY LANGUAGE: EnglishL5 ANSWER 12 OF 29 EMBASE COPYRIGHT 2002 ELSEVIER SCI. B.V.DUPLICATE 4

ACCESSION NUMBER: 1998086348 EMBASE

TITLE: Spermine deficiency in Gy mice caused by deletion of the spermine synthase gene.

AUTHOR: Lorenz B.; Francis F.; Gempel K.; Bsddrich A.; Josten M.; Schmahl W.; Schmidt J.; Lehrach H.; Meitinger T.; Strom T.M.

CORPORATE SOURCE: T.M. Strom, Abteilung Medizinische Genetik, Kinderpöliklinik, Ludwig-Maximilians-Universität, Goethestr. 29, 80336 München, Germany.
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SOURCE: Human Molecular Genetics, (1998) 7/3 (541-547).

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COUNTRY: United Kingdom

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FILE SEGMENT: 022 Human Genetics

LANGUAGE: English

SUMMARY LANGUAGE: English5 ANSWER 13 OF 29 BIOSIS COPYRIGHT 2002 BIOSIS

ACCESSION NUMBER: 1998:227575 BIOSIS

DOCUMENT NUMBER: PREV199800227575

TITLE: Pex mRNA is localized in developing mouse

COMPLETED

Genetic Screening for X-Linked Hypophosphatemic Mice and Ontogenic Characterization of the Defect in the Renal Sodium-Phosphate Transporter

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ABSTRACT

X-linked hypophosphatemic (Hyp) rickets is characterized by short stature, rickets, and bone abnormalities. Biochemically, hypophosphatemia and decreased renal reabsorption of phosphate are the hallmark of the disorder. Mutation of the *PEX* gene has been linked to human and murine Hyp rickets. Our study showed that phenotypical changes of this disease could be detected in 6-wk-old mice, but not in 2-wk-old mice. Therefore, we developed a PCR method to identify Hyp mice by detecting a lack of the 3' region of the *PEX* gene. Serum inorganic phosphate (P_i) levels were decreased, whereas alkaline phosphatase activity was increased in 2- and 6-wk-old Hyp mice. Northern blot showed that renal Na^+-P_i transporter mRNA levels were decreased by 2.1-fold (1.47 ± 0.21 densitometric units for normals; 0.68 ± 1.43 for Hyp mice; $p < 0.040$) in 2-wk-old Hyp mice and by 1.7-fold (2.41 ± 0.42 for normals; 1.44 ± 0.33 for Hyp mice; $p < 0.027$) in 6-wk-old mice. Western blot showed that levels of immunoreactive renal Na^+-P_i transporter protein were decreased

by 4.5-fold (0.90 ± 0.10 for normals; 0.22 ± 0.08 for Hyp mice; $p < 0.001$) in 2-wk-old Hyp mice; and by 4.9-fold (1.47 ± 0.19 for normals; 0.30 ± 0.09 for Hyp mice; $p < 0.0001$) in 6-wk-old Hyp mice. In addition, levels of Na^+-P_i transporter mRNA and protein were increased between 2- and 6-wk-old normal mice, but not in Hyp mice. This study demonstrates an easy assay to detect Hyp mutation and characterizes the defect during ontogeny of the Na^+-P_i transporter in Hyp mice. (*Pediatr Res* 44: 633-638, 1998)

Abbreviations

PEX, phosphate regulating gene with homologies to endopeptidases, on the X chromosome
Hyp, hypophosphatemic
RT, reverse transcription
BBM, brush-border membrane
 P_i , inorganic phosphate

X-linked Hyp rickets is the most common form of inherited vitamin D-resistant rickets in humans. This dominant disorder is characterized by impaired growth and skeletal abnormalities and is manifest as rickets and osteomalacia in children and adults, respectively (1). The hallmark of this disease is hypophosphatemia, secondary to a defect in the renal sodium phosphate (Na^+-P_i) transporter that leads to phosphate wasting (2-4).

The Hyp mouse is an animal model of X-linked Hyp rickets, and it displays biochemical and phenotypical abnormalities similar to the human disease. The defective gene for Hyp was mapped to the short arm of the human X chromosome, Xp22.1-p22.2 (5) and the distal end of the mouse X chromosome (6, 7). Study of this human disease has been greatly facilitated by

discovery of this putative homolog in mouse. However, the mechanisms underlying the bone and renal defects are still unclear. Recent studies have shown that the bone abnormalities are due to low serum phosphate levels, which result from decreased renal phosphate reabsorption (8). However, the intestinal Na^+-P_i transporter is shown to be normal in Hyp mice (9, 10). The defect in Hyp mice has been attributed to a humoral factor that leads to decreased expression of the Na^+-P_i transporter mRNA and protein in the kidney of adult mice (2, 4). However, the ontogenic changes of the renal abnormalities and the developmental progression of this disease are still not known.

The *PEX* gene was identified as the gene defect in Hyp mice and it encodes a protein with homology to neutral endopeptidases (11). Multiple deletion and point mutations have been found in human patients (11, 12). Recently, a 3'-end deletion of the *PEX* gene was found in Hyp mice (13). This observation provides a genetic basis for distinguishing Hyp and normal mice. The current investigation was designed to devise a rapid

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genetic screening test for Hyp mice and to characterize the ontogenic change in the renal $\text{Na}^+\text{-P}_i$ transporter. We developed a simple genomic DNA screening method by PCR to identify Hyp and normal mice. Using this PCR method, we distinguished Hyp from normal mice at 2 and 6 wk of age. We further studied phenotypical changes and expression of the $\text{Na}^+\text{-P}_i$ transporter in normal and Hyp mice during development.

The early detection of Hyp mice by a simple tail-tipped PCR method will significantly enhance our understanding of developmental aspects of vitamin D-resistant rickets. The change of serum P_i level and the defect in the renal $\text{Na}^+\text{-P}_i$ transporter in young Hyp mice without phenotypical changes may provide a unique opportunity for innovative therapies in the early stage of disease.

METHODS

PCR screening of genomic DNA isolated from Hyp and normal mice. Mutant Hyp heterozygote female (C57Bl/6J Hyp⁺, 6 wk) and normal male (C57Bl/6J +/Y, 8 wk) mice were obtained from The Jackson Laboratories (Bar Harbor, ME). The mutant and normal mice were bred in the animal facility at the University of Arizona, College of Medicine. Female offspring mice were killed, and only male mice were used in the following experiments. The genotype of male Hyp and normal offspring of breeding pairs was determined by PCR screening. Body weight and tail length of each animal were determined before the experiments. The tail of the mouse was rinsed in water, then the tip of the tail (0.5 cm) was cut and digested in 700 μL of buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 100 mM NaCl, and 1% SDS) with 17.5 μL of proteinase K (20 mg/mL) at 55°C for 4–5 h. Proteins were extracted with phenol/chloroform, and DNA was precipitated with ethanol. The DNA pellet was dissolved in the 100 μL of H_2O , and 2 μL of genomic DNA were used for subsequent PCR analysis. PCR was carried out using two sets of oligonucleotide primers designed specifically for 5' and 3' regions of the mouse *PEX* gene. The primers for the 5' region of the *PEX* gene (1–105 bp) were: upstream, 5'-TAGCAGACGAGCAAGAGAGT-3'; downstream, 5'-GGCTGGAGAGAAGACTTAGA-3'. The primers for the 3' region of the *PEX* gene (2257–2341 bp) were: upstream, 5'-AGGGTCAATGGTGCCATTAG-3'; downstream, 5'-TGCACCTCTGTTCATAGTGG-3'. The PCR reaction was carried out for 30 cycles as follows: denaturing at 94°C for 1 min, annealing at 59°C for 2 min, and polymerization at 72°C for 2 min. The PCR products were then fractionated by gel electrophoresis on a 3% MetaPhor-agarose gel (FMC BioProducts, Rockland, ME). The normal mice showed both 105- and 84-bp products for the 5' and 3' regions of the *PEX* gene, respectively, whereas Hyp mice only had a PCR product of 105 bp. Both PCR products for the *PEX* gene were subcloned into pGEM-T vectors (Promega, Madison, WI) and confirmed by sequence analysis (Applied Biosystem Division 373 stretch upgraded automated DNA sequencer) and showed 100% identity to the mouse *PEX* cDNA (13).

RT-PCR. Total RNA for RT-PCR was extracted from long bones (femur) of normal and Hyp mice (2 mo old) as described

below. The mutant and normal mice for these experiments were purchased from The Jackson Laboratories. Tissues were excised from animals, snap frozen, homogenized, and placed in a guanidinium thiocyanate solution (4 M guanidinium thiocyanate, 42 mM sodium citrate, 0.83% SDS, and 0.2 mM β -mercaptoethanol in diethyl pyrocarbonate-treated water). Proteins were extracted by phenol/chloroform, and total RNA was precipitated by isopropanol at -20°C . To eliminate contaminating genomic DNA, the RNA sample was digested with RNase-free DNase (10–20 U/100 μg of DNA, Boehringer-Mannheim, Indianapolis, IN) at 37°C for 30 min. After treatment, proteins were extracted by phenol/chloroform, and RNA was precipitated again.

Total RNA (10 μg) from the bone was reversed transcribed using a mixture of random primers (500 ng), oligo(dT) primer (1 μg), RNAsin (40 U), and Moloney murine leukemia virus reverse transcriptase (200 U, Promega) in a total reaction volume of 25 μL . The reaction was performed at 37°C for 1 h; 2.5 μL of the RT mixture was used for subsequent PCR amplification using primers specific for 5' and 3' regions of the *PEX* gene and amplification parameters as described above.

Measurement of serum phosphate and alkaline phosphatase levels. Blood was drawn from the heart of normal and Hyp mice at 2 and 6 wk of age. Serum phosphate levels and alkaline phosphatase activity were measured using Sigma Chemical Co. diagnostic kits (Sigma Chemical Co., St. Louis, MO), and the procedures were done following the manufacturer's protocol. The reaction of phosphate with ammonium molybdate in the presence of H_2SO_4 produced a phosphomolybdate complex. The absorbance of this complex at 340 nm was measured by a spectrophotometer, and this value was used to calculate P_i levels. To assess serum alkaline phosphatase activity, serum was incubated with *p*-nitrophenyl phosphate, and the absorbance of converted complex was measured at 400–420 nm. This value was used to calculate alkaline phosphatase activity.

Northern blot analysis of renal $\text{Na}^+\text{-P}_i$ transporter mRNA levels. Total RNA was isolated from kidney cortex of individual normal and Hyp mice at 2 and 6 wk of age as described above. Total RNA (15 μg) from each animal was used for Northern blot analysis by a standard technique as described previously (14, 15). RNA was fractionated by denaturing agarose gel electrophoresis, and transferred to a nitrocellulose membrane. The membrane was prehybridized at 65°C for 4 h with a buffer containing 6 \times SSC, 5 \times Denhardt's solution, 10% dextran sulfate, 1% SDS, and 100 $\mu\text{g}/\text{mL}$ denatured salmon sperm DNA. Hybridization was carried out at 65°C overnight in the same solution with [α - ^{32}P]dCTP-labeled probes prepared from full-length mouse $\text{Na}^+\text{-P}_i$ transporter type II cDNA (13) by random prime labeling (Amersham, Arlington Heights, IL; specific activity, 1×10^7 dpm/mL). High stringency washes were carried out at 65°C with 0.1 \times SSC and 0.1% SDS. The blot was subsequently reprobed with 1B15 cDNA-specific probe, which is a constitutively expressed gene encoding rat cyclophilin (14). The blot was imaged and the signal intensities were measured by phosphorimage analysis (GS-525, Bio-Rad). Hybridization intensities for each sample were normalized for 1B15 levels on the same blot.

Western blot analysis of the Na⁺-P_i transporter protein levels. Kidney cortex was excised from 2- and 6-wk-old normal and Hyp mice. BBMs were purified by the MgCl₂ precipitation technique as described previously (2). Protein was quantitated by Lowry protein assay (Bio-Rad kit). Purity and enrichment of one kidney cortex membrane preparation was assayed by measuring a BBM-specific marker, leucine aminopeptidase activity (Sigma Chemical Co. diagnostic kit). Protein samples (20 µg) were fractionated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked overnight in PBS/0.05% polyoxyethylene sorbitan monolaurate (Tween 20)/5% powdered milk. The strips were then incubated with primary antiserum at 1:4000 dilution in PBS/0.05% Tween 20/0.1% milk at room temperature for 1 h. This polyclonal antibody was raised in rabbit against a mouse Na⁺-P_i transporter-specific COOH-terminal peptide, and the specificity of this antiserum for Na⁺-P_i transporter has been extensively characterized previously (2, 14, 15). After several more washes, the strips were incubated with secondary antibodies (anti-rabbit immunoglobulin horseradish peroxidase-linked antibody) at 1:1500 dilution for 45 min at room temperature. After several washes, the strips were exposed to enhanced chemiluminescence reagents (ECL system; Amersham) for 1 min and placed to film for 30 s to 3 min. Subsequently, the blots were reacted with mouse monoclonal β-actin antiserum (Sigma Chemical Co.), which serves as an internal control for gel loading and transfer. Signal intensities for the Na⁺-P_i transporter were determined by densitometric analysis using the Bio-Rad GS-700 image densitometer and were normalized for β-actin signal intensities on the same blot.

Statistical analysis of results. All data were analyzed for statistical significance by factorial one-way ANOVA, using the StatView software (Abacus Concepts, Inc., version 4.53) and are presented as mean ± S.E.M.

RESULTS

Identification of normal and Hyp mice by PCR amplification of genomic DNA. To determine whether animals were normals or mutants, genomic DNA isolated from the tip of the mouse tail was used for PCR amplification. Figure 1A shows PCR amplification products from 2- and 6-wk-old normal and Hyp mouse genomic DNA using specific primers for 5' and 3' regions of the *PEX* gene. The expected band size for the 5' region of the *PEX* gene is 105 bp and for the 3' region is 84 bp. The results showed that both PCR products were obtained from normal mice and only the PCR product for the 5' region of the *PEX* gene was obtained in Hyp mice. This lack of the 3' region of the *PEX* gene in Hyp mice was further confirmed by RT-PCR amplification of cDNA generated from bone mRNA in which the *PEX* gene mRNA is expressed. Figure 1B shows that both bands were amplified from the bone RT reaction of normal mice, but only the 5' region of the *PEX* gene was amplified from the bone RT reaction of Hyp mice. These results are consistent with the previous observation that 3' region of the *PEX* gene was deleted in Hyp mice (13). Based on this deletion mutation in Hyp mice, the PCR detection of the *PEX* gene mutation from genomic DNA allowed us to identify

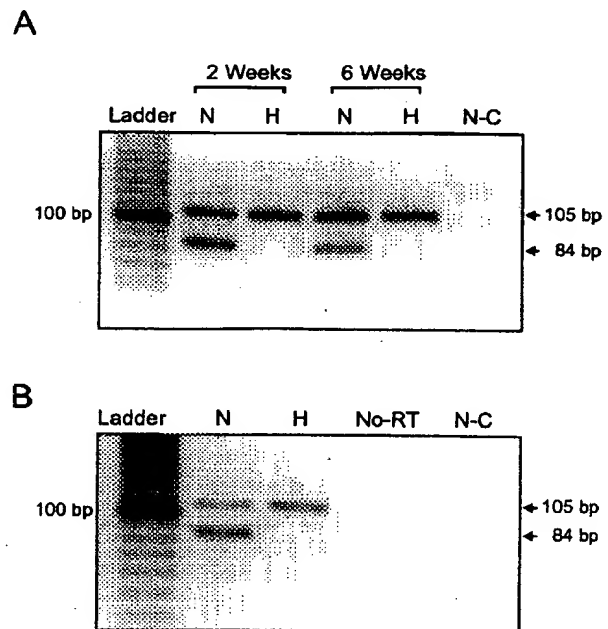


Figure 1. Identification of normal and Hyp mice by PCR amplification. (A) Genomic DNA was isolated from the tip of the tail of individual normal (N) and Hyp (H) mice at 2 and 6 wk of age. PCR amplification was performed with primers specific for 5' and 3' regions of the *PEX* gene in separate reactions. PCR products from these two reactions (105 bp for the 5' region and 84 bp for the 3' region) were loaded in the same gel lane for each sample. N-C is negative PCR amplification without template DNA. (B) Total RNA was extracted from bone of normal (N) and Hyp (H) of adult mice (2 mo). PCR amplification was carried out using cDNA reverse transcribed from RNA. RT-PCR products for 5' and 3' regions of the *PEX* gene were loaded in the same gel lane. No-RT is PCR amplification using RNA as a template. N-C is a PCR amplification without DNA.

the normal and Hyp mice at any developmental stage for subsequent experiments.

Phenotypical changes in Hyp mice at 2 and 6 wk of age.

Figure 2 shows the phenotypical changes in normal and Hyp mice at 2 and 6 wk of age. The body weight and tail length measured from each animal were compiled in Figure 2, A and B, respectively. In 2-wk-old animals, data points overlapped between normal and Hyp mice, and there are no significant differences in body weight or tail length between the two groups. The body weight was 8.23 ± 0.26 g for normals ($n = 14$) and 7.76 ± 0.41 g for Hyp mice ($n = 11$); tail length was 3.99 ± 0.08 cm for normals ($n = 14$) and 3.72 ± 0.10 for Hyp mice ($n = 11$). However, in 6 wk old Hyp mice, the body weight and tail length were significantly decreased as compared with normal mice. Body weight of 6-wk-old mice was 22.04 ± 0.38 g for normal mice ($n = 12$) and 18.53 ± 0.36 g for Hyp mice ($n = 11$; $p < 0.0001$). Tail length for 6-wk-old animals was 7.23 ± 0.06 cm for normal ($n = 12$) and 5.58 ± 0.08 cm for Hyp mice ($n = 11$; $p < 0.001$). These data indicated that Hyp mice did not develop any phenotypical changes at the early stage (2 wk), but manifest a shorter tail and lower body weight at the later stage (6 wk).

Changes in serum phosphate levels and alkaline phosphatase activity in 2- and 6-wk-old Hyp mice. Serum phosphate

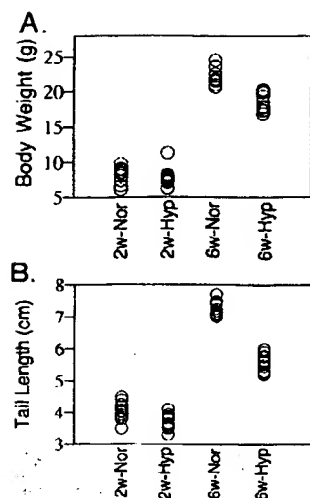


Figure 2. Assessment of phenotypical changes in body weight and tail length in 2- and 6-wk-old Hyp mice. Body weight and tail length were measured from normal and Hyp mice at 2 and 6 wk of age. Data for body weight and tail are compiled in panels A and B, respectively. The graphs show the distribution of data points obtained from individual animals. 2w-Nor and 6w-Nor are 2- and 6-wk-old normal mice, respectively. 2w-Hyp and 6w-Hyp are 2- and 6-wk-old Hyp mice, respectively. The mean \pm SEM is indicated only in the text.

(P_i) levels and alkaline phosphatase activity from normal and Hyp mice at 2 and 6 wk of age are depicted graphically in Figure 3, A and B, respectively. In 2-wk-old animals, serum P_i levels were 14.62 ± 0.51 (mg/dL) for normals ($n = 14$) and 10.10 ± 0.61 mg/dL for Hyp mice ($n = 7$). In 6-wk-old mice, serum P_i levels were 11.78 ± 0.46 mg/dL for normals ($n = 11$) and 6.81 ± 0.25 mg/dL for Hyp mice ($n = 11$). Interestingly, although there are no phenotypical changes in 2-wk-old Hyp mice as described above, serum P_i levels were significantly decreased in both 2- and 6-wk-old Hyp mice ($p < 0.0001$ for

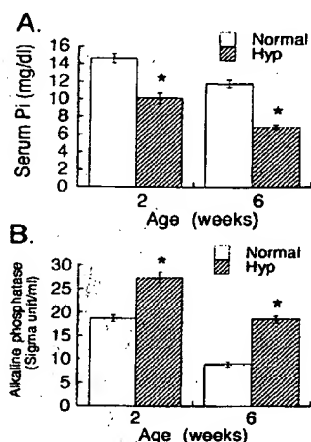


Figure 3. Changes in serum P_i levels and alkaline phosphatase activity in 2- and 6-wk-old Hyp mice. Serum P_i levels (A) and alkaline phosphatase activity (B) were measured from normal and Hyp mice at 2 and 6 wk of age. Data are presented as mean \pm SEM for each group. The asterisk (*) indicates a significant difference between normal and Hyp mice for both ages ($p < 0.0001$). In addition, for normal mice, there are significant differences between 2- and 6-wk groups for both serum P_i levels and alkaline phosphatase activity ($p < 0.0001$ for both, not indicated).

both ages, normal versus Hyp). For serum alkaline phosphatase (Fig. 3B), activity levels were 18.73 ± 0.78 U/mL (Sigma Chemical Co.) for normals and 27.42 ± 1.13 U/mL for Hyp mice at 2 wk of age, and were 8.86 ± 0.56 U/mL for normals and 18.51 ± 0.80 U/mL for Hyp mice at 6 wk of age. These values showed significant differences between normal and Hyp mice at both age groups ($p < 0.0001$, normal versus Hyp for both ages). These data indicated that serum P_i levels and alkaline phosphatase activity were significantly altered in the young Hyp mice, although they have not yet developed any phenotypical changes. In addition, serum P_i levels were significantly decreased between 2- and 6-wk-old animals in both normal and Hyp groups ($p < 0.0001$ 2 wk versus 6 wk for both), whereas alkaline phosphatase activity was significantly decreased between 2- and 6-wk-old mice in both normals and Hyp mice ($p < 0.001$, 2 wk versus 6 wk for both).

Changes in Na^+-P_i transporter mRNA levels in Hyp mice at 2 and 6 wk of age. The defect in the renal Na^+-P_i transporter expression pathway in 2- and 6-wk-old Hyp mice was studied at the levels of mRNA and protein. Northern blot analysis was used to determine the level of Na^+-P_i transporter mRNA in 2- and 6-wk-old normal and Hyp mice (Fig. 4). The 2.6-kb band is specific for the renal Na^+-P_i transporter and the 1.0-kb band corresponds to rat 1B15 (Fig. 4A). In addition, a faint 4.6-kb band was also hybridized with the Na^+-P_i probe as previously described (2) (data not shown). The signal intensity of the Na^+-P_i transporter was normalized by the 1B15 signal for each sample. The data were averaged and presented graphically in

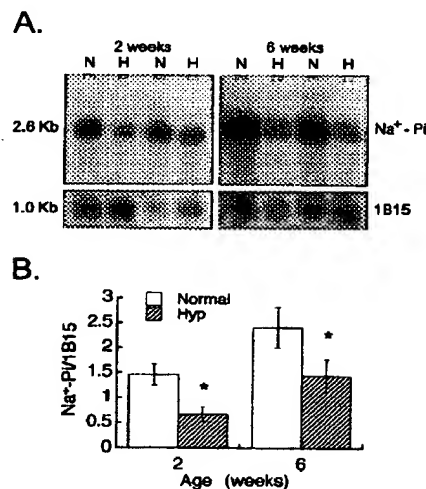


Figure 4. Northern blot analysis of renal Na^+-P_i transporter mRNA levels in normal and Hyp mice at 2 and 6 wk of age. (A) Total RNA was prepared from kidney cortex of individual normal (N) and Hyp (H) mice at 2 and 6 wk of age. Hybridization to mouse renal Na^+-P_i transporter-specific probes showed a predominant transcript at 2.6 kb and a minor transcript at 4.6 kb (the 4.6-kb band was revealed only after long exposure; data not shown). The hybridization signal at 1.0 kb corresponds to a constitutive 1B15 (cyclophilin) on the same blot. (B) Signal intensity for the Na^+-P_i transporter was normalized by signal intensity for 1B15, and data are presented as mean \pm SEM for each group. The asterisk (*) indicates a significant difference between normal and Hyp mice in both age groups ($p < 0.03$ for 2 wk; $p < 0.02$ for 6 wk). For normal mice, there is also a significant difference between 2 and 6 wk ($p < 0.02$; not indicated).

Figure 4B. The results showed that $\text{Na}^+\text{-P}_i$ transporter mRNA levels in 2-wk-old mice were 1.47 ± 0.21 arbitrary densitometric units in normals ($n = 6$) and 0.68 ± 0.14 in Hyp mice ($n = 7$), whereas for 6-wk-old mice levels were 2.41 ± 0.42 in normals ($n = 5$) and 1.43 ± 0.33 ($n = 5$) in Hyp mice. Statistical analysis showed significant decreases in both 2- and 6-wk-old Hyp mice as compared with normal mice ($p < 0.039$, normal versus Hyp at 2 wk; $p < 0.027$, normal versus Hyp at 6 wk). In addition, in normal mice, the $\text{Na}^+\text{-P}_i$ transporter mRNA was significantly increased during ontogeny ($p < 0.025$, 2 versus 6 wk). Although statistical analysis showed no significance difference between ages in Hyp mice ($p = 0.058$), there was a strong trend that $\text{Na}^+\text{-P}_i$ transporter mRNA levels were developmentally increased in Hyp mice. These data demonstrated that a defect in the renal $\text{Na}^+\text{-P}_i$ transporter at the mRNA level in Hyp mice occurred at least as early as 2 wk.

Changes in $\text{Na}^+\text{-P}_i$ transporter protein levels in Hyp mice at 2 and 6 wk of age. Kidney BBM were obtained from individual mice. The membrane preparation was enriched 22–33-fold compared with crude membrane homogenate as determined by leucine aminopeptidase activity. Figure 5A shows Western blot analysis of renal BBM proteins from normal and Hyp mice at 2 and 6 wk of age. Antiserum specific for mouse renal $\text{Na}^+\text{-P}_i$ transporter showed a predominant 87-kD band, and antiserum against β -actin showed a 42-kD band. In addition, a minor 37-kD band was also detected with antiserum against the $\text{Na}^+\text{-P}_i$ transporter as previously described (2, 14) (data not shown). The intensity of the signal for the 87-kD $\text{Na}^+\text{-P}_i$ transporter band was normalized by β -actin on the

same blot, and results are depicted graphically in Figure 5B. For 2-wk-old animals, the signal intensity was significantly decreased in Hyp mice (0.22 ± 0.08 densitometric units, $n = 5$) as compared with normal mice (0.90 ± 0.10 , $n = 7$; $p < 0.0011$). For 6-wk-old animals, the signal intensity was also significantly decreased in Hyp (0.30 ± 0.09 , $n = 5$) compared with normal (1.47 ± 0.19 , $n = 6$; $p < 0.0001$) mice. The normal mice showed ontogenic increase of $\text{Na}^+\text{-P}_i$ protein levels from 2 to 6 wk ($p < 0.0001$). However, Hyp mice showed no significant difference during development.

DISCUSSION

X-linked hypophosphatemic rickets shows similar phenotypic changes in humans and mice, including growth retardation and skeletal abnormalities. In 6-wk-old mice, a 16% reduction in body weight and a 23% shortening in tail length were observed in Hyp mice compared with normal mice. However, these changes were not evident in the early age (suckling stage, 2 wk), suggesting that the skeletal abnormalities develop over a period of time and are manifest only in the older age group. Therefore, there were no phenotypic assessments that could be used in the early stage to distinguish Hyp from normal mice. Here we report a simple tail-tipped PCR method to screen for the genotype of male Hyp and normal mice. Using PCR to amplify genomic DNA isolated from mice allows us to accurately and rapidly segregate the normal and mutant (Hyp) mice in the male sex group. Both 5' and 3' regions of the *PEX* gene were amplified from normal mice, whereas only 5' region of the *PEX* gene was amplified from Hyp mice, because a uniform deletion mutation has been identified in all Hyp mice (13). This method, however, cannot distinguish normal from heterozygote female animals, because most studies were carried out using male mice. This PCR method facilitated the studies of Hyp mice, particularly in the early stage when the phenotypic changes were not evident.

To further confirm this method, mice screened at 2 wk of age were followed into adulthood and physiologic parameters were assessed. We found that Hyp or normal mice that were genotyped by PCR at 2 wk of age indeed developed Hyp or normal phenotype in adulthood as expected.

Serum P_i levels and alkaline phosphatase activity were measured in 2- and 6-wk-old mice. Decreased serum P_i levels and increased alkaline phosphatase activities in adult Hyp mice have been shown previously (1, 17). The current study additionally showed that, in 2-wk-old animals, Hyp mice have already developed these biochemical changes. The serum P_i level was decreased by 31% in 2-wk-old Hyp mice, whereas a reduction of 42% was observed in 6-wk-old mice. In contrast to serum P_i level, the activity of alkaline phosphatase was increased by 46% in 2-wk-old Hyp mice and 109% in 6-wk-old Hyp mice compared with the normals. Although the Hyp state occurred in the early stage (2 wk), the phenotypic features of Hyp mice were not evident until the later stage (6 wk), suggesting that the bone abnormalities resulted from low serum P_i levels and took place over a period of time.

The pathogenesis of the hypophosphatemic rickets has been linked to a defect in the renal $\text{Na}^+\text{-P}_i$ transporter (2–4, 15).

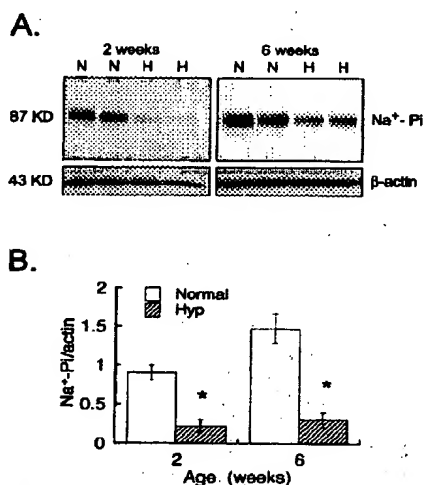


Figure 5. Western blot analysis of renal $\text{Na}^+\text{-P}_i$ transporter protein levels in normal and Hyp mice at 2 and 6 wk of age. (A) BBM proteins were isolated from individual normal (N) and Hyp (H) mice at 2 and 6 wk. Protein (20 μg) from each sample was fractionated by 10% SDS-polyacrylamide gel and transferred onto nitrocellulose. Reaction with a mouse specific $\text{Na}^+\text{-P}_i$ transporter antiserum shows a predominant 87-kD band and a minor 33-kD band (33-kD band not shown). β -Actin serves as an internal control for loading. (B) Signal intensity for the $\text{Na}^+\text{-P}_i$ transporter was normalized by signal intensity for β -actin, and data are presented as mean \pm SEM for each group. The asterisk (*) indicates a significant difference between normal and Hyp mice in both age groups ($p < 0.001$ for 2 wk; $p < 0.0001$ for 6 wk). For normal mice, there is also a significant difference between ages of 2 and 6 wk ($p < 0.003$; not indicated).

Previous studies have shown a decrease in renal BBM vesicle sodium-dependent phosphate uptake in Hyp mice (3, 9). Molecular characterization of this defect showed a 3-fold decrease in mRNA level for the Na^+-P_i transporter and 6–10-fold decrease in protein levels in adult Hyp mice (2, 15). However, whether this Na^+-P_i transporter defect occurs in the early stage of Hyp mice is not known. The present study showed that Na^+-P_i transporter mRNA levels were decreased by 2.1-fold in 2-wk-old Hyp mice and 1.7-fold in 6-wk-old Hyp mice as determined by Northern blot analysis. Western blot analysis showed that Na^+-P_i transporter protein levels were decreased by 4.5-fold in 2-wk-old Hyp mice and 4.9-fold in 6-wk-old mice. The discrepancy of decreased levels of the Na^+-P_i transporter mRNA and proteins in older mice between the current and previous studies mentioned above may be due to the fact that individual animals were used here as opposed to groups of animals in the previous study, or that 6-wk-old animals were used in the current study, whereas 6–10-wk-old animals were used in the previous study. Overall, in the current study, the reduction of the Na^+-P_i transporter protein is greater than mRNA, suggesting that a posttranscriptional mechanism in addition to a transcriptional mechanism may be involved in regulation of renal Na^+-P_i transporter in Hyp mice. To date, there are no other studies that have been done to address this question.

Ontogenic changes in renal Na^+-P_i transporter expression were studied only in rats previously. The abundance of renal Na^+-P_i transporter mRNA analyzed by Northern blot was not significantly changed during development in the rat. However, the immunoreactive protein level analyzed by Western blot analysis showed a gradual increase from 2 wk of age to adulthood (18). However, the current study showed that expression of the Na^+-P_i transporter mRNA was regulated during development in normal mice. In normal mice, the mRNA level for the Na^+-P_i transporter was significantly increased by 1.7-fold, whereas the protein level was significantly increased by 1.6-fold between 2 to 6 wk of age. The correlated increases in levels of mRNA and protein during ontogeny of normal mice suggested a transcriptional regulation may be involved. However, it is still unclear why mice and rats showed different developmental expression patterns; that Na^+-P_i mRNA levels were up-regulated in mice, but did not change in rats.

The *PEX* gene, a candidate gene for X-linked hypophosphatemic rickets, has been known to be expressed predominantly in the mouse bone, lung, and embryo (13). The early expression of this gene may dictate an early onset of regulation of the renal Na^+-P_i transporter in mice. In Hyp mice, a large 3'-end deletion (approximately 1 kb) of the *PEX* gene transcript has been identified (13). Therefore, most likely, this genetic defect affects the expression of the Na^+-P_i transporter in the early stage of development. This hypothesis was indeed confirmed by an early occurrence of the renal Na^+-P_i transporter defect in genetically identified Hyp mice observed in our study. The inhibition of expression of Na^+-P_i transporter and changes in serum P_i and alkaline phosphatase were detected in 2-wk-old Hyp mice. These pathologic changes in Hyp mice possibly occur even earlier than the 2 wk of age studied here.

However, phenotypical changes were developed only after 2 wk of age. Significant growth retardation as assessed by lower body weight and shorter tail length in Hyp mice can be identified in 6-wk-old Hyp mice. These results demonstrate the great potential and implications in seeking early intervention for human patients whose serum P_i levels are already reduced, but have not yet developed phenotypes. The early effective therapy in raising serum P_i levels may eventually prevent the skeletal abnormalities in the later stage of disease. However, conventional therapies with phosphate and vitamin D have not been effective, and future investigations should be directed at developing gene therapy strategies at the early stage. Osteoblast cell-specific delivery of the normal *PEX* gene or direct delivery of Na^+-P_i gene to renal cortex of Hyp mice by intrarenal-pelvic infusion at the early stage will be suitable approaches to correct the defect in Hyp mice.

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AUTHOR: Carpenter T.O.

CORPORATE SOURCE: Dr. T.O. Carpenter, Department of Pediatrics, Yale University School of Medicine, New Haven, CT 06520-8064, United States

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TITLE: Per gene deletions in Gy and Hyp mice provide mouse models for X-linked hypophosphatemia.

AUTHOR: Strom T.M.; Francis F.; Lorenz B.; Boddrich A.; Econs M.J.; Lehrach H.; Meitinger T.

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ARTICLE

Pex gene deletions in Gy and Hyp mice provide mouse models for X-linked hypophosphatemia

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X-linked hypophosphatemic rickets in humans is caused by mutations in the *PEX* gene which codes for a protein homologous to neutral endopeptidases. Hyp and Gy mice both have X-linked hypophosphatemic rickets, although genetic data and the different phenotypic spectra observed have previously suggested that two different genes are mutated. In addition to the metabolic disorder observed in Hyp mice, male Gy mice are sterile and show circling behavior and reduced viability. We now report the cloning of the mouse homolog of *PEX* which is highly conserved between man and mouse. The 3' end of this gene is deleted in Hyp mice. In Gy mice, the first three exons and the promotor region are deleted. Thus, Hyp and Gy are allelic mutations and both provide mouse models for X-linked hypophosphatemia.

INTRODUCTION

A gene mutated in patients with X-linked hypophosphatemic rickets recently has been described by the HYP consortium (1). The gene named *PEX* (phosphate-regulating gene with homologies to endopeptidases on the X chromosome) was identified by positional cloning and is predicted to encode a protein which belongs to the neutral endopeptidase family of zinc metallo-proteinases including neutral endopeptidase (NEP), endothelin-converting enzyme 1 and 2 (ECE) and Kell antigen (2-6). Loss-of-function mutations are present in a large proportion of familial and sporadic cases of hypophosphatemia (Francis *et al.*, in preparation). *PEX* may activate a phosphate-conserving protein or deactivate a phosphate-wasting protein, but the exact functional role of the endopeptidase remains obscure.

Two mouse models for hypophosphatemia have been described extensively, Hyp (7) and Gy (8) mice. Hyp arose as a spontaneous mutation and affected mice show hypophosphatemia, impaired renal tubular phosphate reabsorption and vitamin D non-responsive rickets or osteomalacia, closely resembling human X-linked hypophosphatemic rickets (7). The Gy mutation was found among the offspring of an irradiated female mice. In addition to hypophosphatemia, affected Gy males and some affected female animals show circling behavior and inner ear abnormalities. Furthermore, male affected animals are sterile and have reduced viability from birth, with sudden death occurring in adults (8). There has been speculation that a subset of X-linked HYP patients

with hearing defects may be the counterpart of the Gy phenotype in mouse (9).

Both Hyp and Gy loci are known to map to a region of the mouse X chromosome syntenic to the human *PEX* locus. They have been reported to show a recombination fraction of 0.4-0.8% (8). This led to the suggestion that mutations in two different genes, closely situated on the X chromosome and both involved in phosphate metabolism, are responsible for hypophosphatemia.

There have been conflicting reports regarding the biochemical differences between the Hyp and Gy mouse phenotypes. Gy mice have been reported to maintain an appropriate elevation of renal 1 α -hydroxylase relative to hypophosphatemia (10), while Hyp mice manifest only normal, not increased, enzyme activity (11-13). However, elevation of renal 1 α -hydroxylase levels in Gy mice could not be confirmed by other investigators (14). Two types of renal sodium phosphate co-transporters have been cloned (15,16) and reduced mRNA levels for the type 2 sodium phosphate co-transporter in Hyp (17) and Gy (18) kidney have been demonstrated. Other investigators, however, found that mRNA levels for this co-transporter were normal although the levels of immunoreactive protein were reduced (19). This led to the proposition that the molecular defects in the expression of this co-transporter are distinct in Hyp and Gy mice.

There has also been speculation that Hyp and Gy exhibit strain-related differences since they are present on different background strains. Hyp mice are maintained on a C57BL/6J

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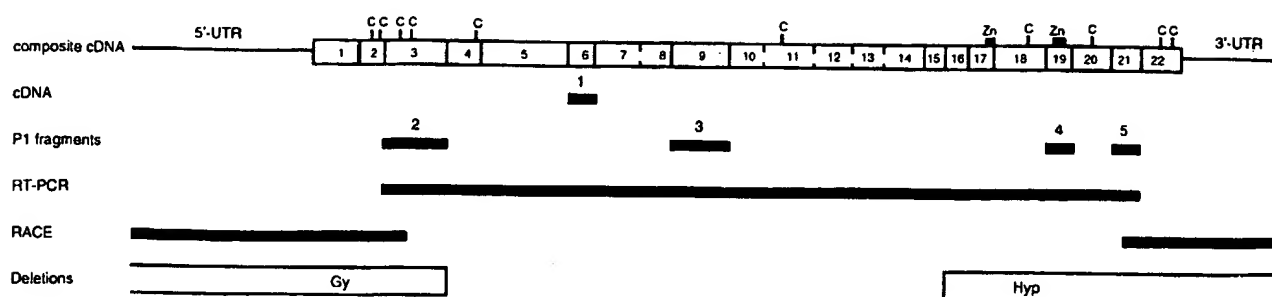


Figure 1. Cloning strategy of the *Pex* gene and mapping of intragenic deletion breakpoints in Hyp and Gy. The composite cDNA sequence was derived from (1) an unspliced spleen cDNA clone, (2) a 2.9 kb *EcoRI*–*HindIII* fragment, and (3) a 2.5 kb *HindIII* fragment of P1 703H24279, (4) a 1.3 kb *EcoRI* fragment and (5) a 1.0 kb *EcoRI* fragment of P1 703B06300. RT-PCR and RACE reactions were then performed to complete the sequence. The exon boundaries marked by a solid line were derived from the genomic sequence or by assigning RT-PCR products of incremental exon length (based on the knowledge of the exon boundaries in the human gene) to *EcoRI* or *HindIII* bands. Exon boundaries marked by dotted lines are not yet determined and are drawn corresponding to those in the human gene. Gy bears a deletion of the first three exons including the 5'-UTR. In Hyp, the last seven exons including the 3'-UTR are deleted. C = conserved cysteines, Zn = zinc coordinating domains.

background, whereas Gy mice are on a B6C3H background. When the Gy mutation is transferred to the C57BL/6J strain, however, male Gy mice do not survive (20). Therefore, it seems that interstrain differences alone do not explain the phenotypic and biochemical differences. In summary, it has been widely assumed that Hyp and Gy are different but closely linked loci.

Recently, the sequence of the mouse homolog of *PEX* and its expression in bone tissue has been described by Du *et al.* (21). No mutations were detected in Hyp mice using a RT-PCR approach, although bone tissue and cultured osteoblasts were found to be negative for *Pex* expression. Here we report allelic *Pex* mutations in both Hyp and Gy, thus providing mice models for the human disease.

RESULTS

Mouse homolog of *PEX*

To obtain the mouse homolog of *PEX*, we first screened 1 000 000 clones each of a mouse spleen and mouse B-cell library. Only a single unspliced clone (2.9 kb) was obtained from the spleen library corresponding to bp 664–732 (corresponding to human exon 6) of the final mouse sequence.

We then screened a mouse P1 library with human *PEX* cDNA and isolated two clones 703H24279 and 703B06300. Clone 703H24279 was positive for a cDNA fragment corresponding to bp 1–1079 of the composite mouse sequence, clone 703B06300 to a cDNA fragment corresponding to bp 1900–2148. Positive fragments of the P1 clones (2.9 kb *EcoRI*–*HindIII* and 2.5 kb *HindIII* fragments of 703H24279 and 1.3 and 1.0 kb *EcoRI* fragments of 703B06300) were subcloned and sequenced. The 2.9, 2.5, 1.3 and 1.0 kb fragments contained the sequence from bp 191 to 349 (corresponding to human exon 3), 934–1079 (corresponding to human exon 9), 1900–1968 (corresponding to human exon 19) and 2071–2148 (corresponding to human exon 21), respectively. The sequence between these exons was obtained by RT-PCR in mouse cDNA randomly transcribed from white blood cells, 12.5 day total fetal mouse and mouse adult kidney RNA.

To obtain the 5' and 3' end of the gene, we used rapid amplification of cDNA ends (RACE) with 17 day total fetal mouse cDNA as template. PCR fragments of ~800 and 900 bp

were generated by 5' RACE and 3' RACE, respectively. Sequencing of the 5' RACE product yielded the missing 5' coding sequence and 530 nucleotides of the upstream sequence. The 3' RACE product yielded the missing 3' coding sequence, 216 bp of the downstream sequence and ended in a CTTT repeat (Fig. 1).

RT-PCR was performed successfully in cDNA from mouse bone, muscle and fetal brain tissues.

Gene structure

The composite cDNA sequence comprises 2993 nucleotides. The predicted open reading frame (ORF) of 2247 nucleotides encodes a protein of 749 amino acids. The gene is highly conserved between man and mouse. The mouse gene shows 91% identity at the DNA level and 96% identity at the protein level to the human gene (GenBank accession no. U60475). The sequence identity is lower in the intracellular and membrane domain (83% between amino acids 1 and 58) than in the extracellular part (97% between 59 and 749) of the proteins (Fig. 2).

We assigned the start codon to the first AUG codon after an in-frame stop codon at position –24 to –21. The sequence around this start codon deviates from the Kozak consensus sequence especially in the –3 position (22), where a purine residue is expected. Also, the 5'-untranslated sequence is burdened with two in-frame and three out-of-frame AUG codons. These deviant features have only been found in genes which are highly regulated at the post-transcriptional level and thus intended for poor translation. On the other hand, these phenomena have been reported in cDNAs which do not correspond to the mature mRNA but are caused by alternative splicing or promotor switching (23). However, within 11 kb of genomic sequence upstream of the human gene (Francis *et al.*, in preparation) there was no indication of additional exons or promotor sites predicted by exon recognition programs. Furthermore, the 530 nucleotides of the 5'-untranslated sequence of *Pex* show a high identity of 82% with the human sequence, and the first seven translated amino acids (MEAETGS) are identical, indicating that the AUG at position 1–3 could in fact correspond to the start codon.

With the cloning of the mouse homolog of *PEX*, the complete cDNA sequence is now available. Similarity searches confirm that *Pex* is a member of the endopeptidase (24) or M13 family of metallopeptidases (25) which comprises NEP, ECE-1, ECE-2

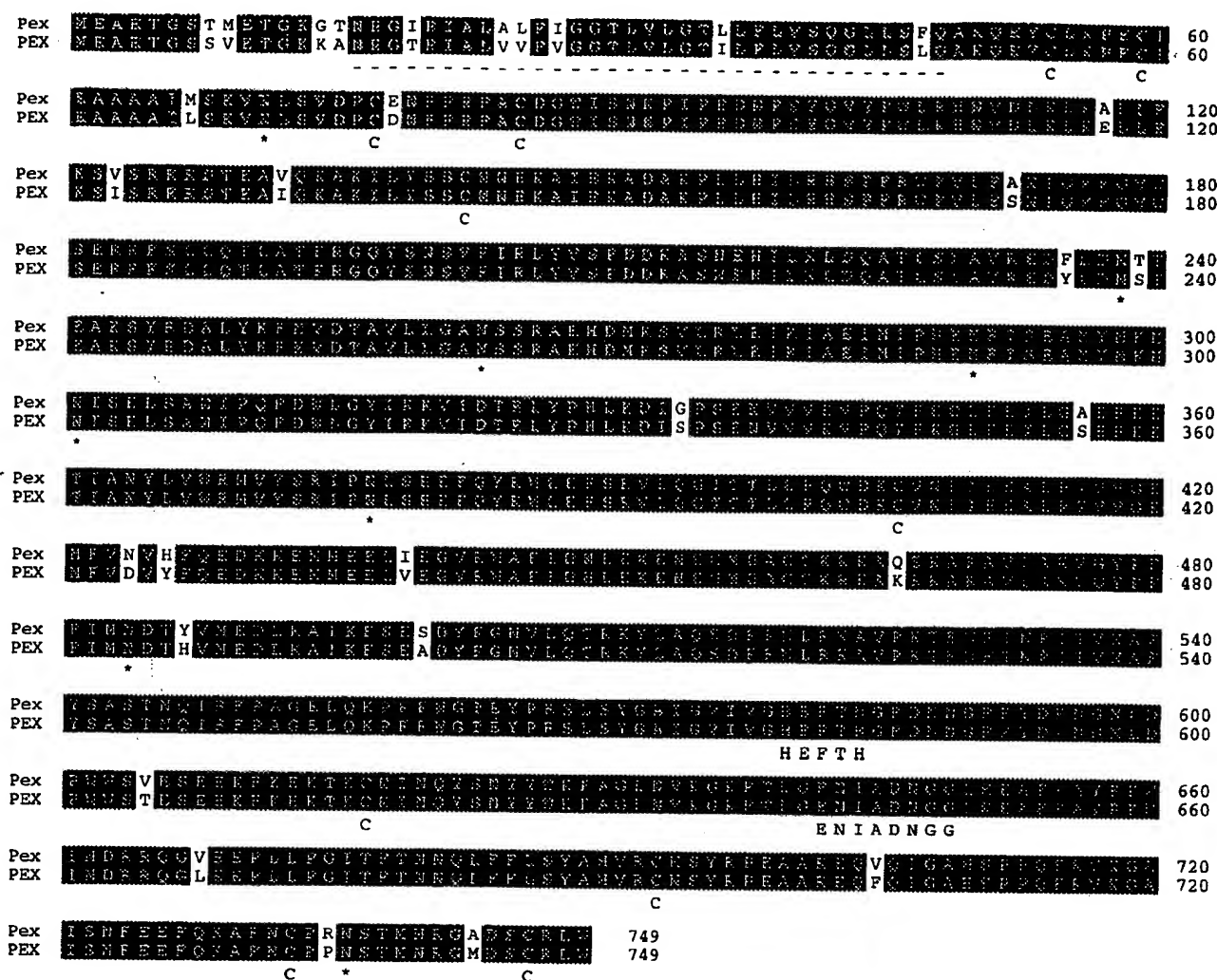


Figure 2. Alignment of PEX with the mouse homolog Pex generated with CLUSTAL and PRETTYBOX (GCG version 8.1). The dashed line indicates the predicted transmembrane domain. The potential Asn glycosylation sites are indicated by asterisks, the cysteine residues which are conserved between NEP, ECE, KELL and PEX by a C, and the zinc-coordinating motifs by the sequences HEFTH and ENIADNGG.

and KELL and show an overall similarity of 50–60% between each other. The extracellular domain of Pex contains 10 cysteine residues that are conserved within the endopeptidase family and suggest not only primary but also tertiary structure similarity within this family. The homology is more pronounced in the C-terminal fifth (275 amino acids) of the gene which contains the zinc-coordinating motifs. At position 580–584, Pex contains the zinc binding motif which is characteristic for most zincins (HEXXH) (26) where the two histidines are zinc coordinating. Sixty three amino acids downstream lies the motif which is characteristic for the endopeptidase family (ENXADXGG) (27) where glutamic acid is the third zinc ligand. In the case of NEP, amino acids involved in substrate binding have been identified using site-directed mutagenesis. Glu584 and Asp650 participate in the catalytic mechanism (27,28). His711 was shown to be involved in the stabilisation of the tetrahedral intermediate during the transition state (29). These amino acids are conserved in Pex

and correspond to Glu581, Asp645 and His710.

Hyp and Gy deletions

In addition to the five *Pex* exons for which the exon–intron boundaries have been sequenced, the overall exon–intron structure was characterised by assigning *Pex* exons to single *EcoRI* and *HindIII* fragments. Overlapping RT-PCR products corresponding to human exons 1–5 identified 4.5 (exon 1), 11 (exons 2 and 3) and 4.5 kb (exons 4 and 5) *EcoRI* fragments and 2.7 (exon 1), 5.9 (exon 2), 6.1 (exon 3) and >15 kb (exons 4 and 5) *HindIII* fragments in control mice. Overlapping RT-PCR products corresponding to human exons 15–22 identified 6.0 (exon 15), 6.5 (exon 16), 4.0 (exons 17 and 18), 1.3 (exon 19), 2.1 (exon 20), 1.0 (exon 21) and 4.5 kb (exon 22) *EcoRI* fragments in control mice. Three *EcoRI* fragments (6, 8.1 and >20 kb) were identified by hybridising a RT-PCR product which spans exons 12–15.

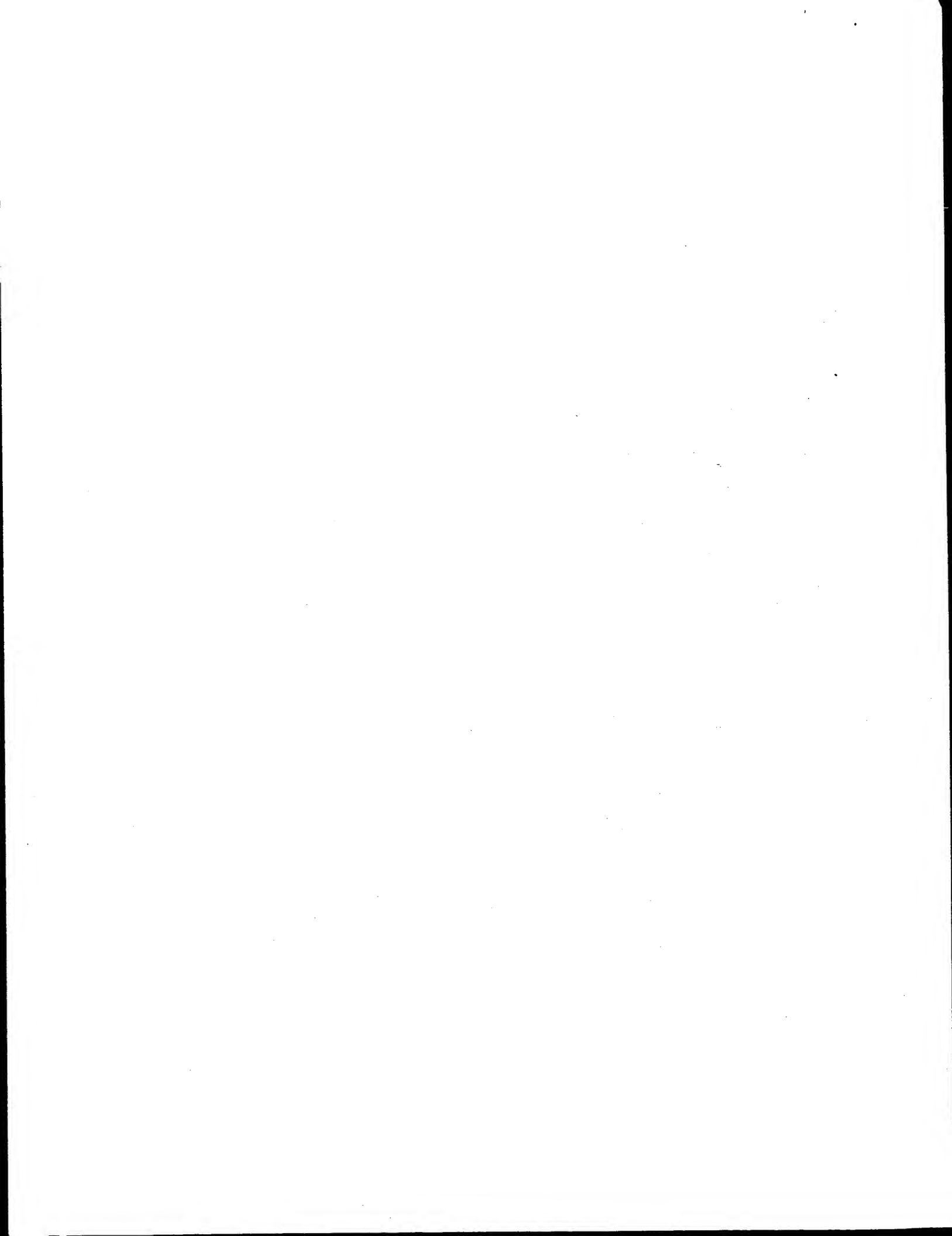


Table 1. Primers used for amplification and sequencing *Pex* exons

Exon ^a	Primer A	Primer B	bp	°C
3	5'-TCTTGTCACACAGTGTCTGG-3'	5'-CCAGGGAGACATTTGAGGAG-3'	300	60
6	5'-TGGAGTGAACCTATTCTTGGG-3'	5'-ACCAAACAGTAGCCAGACAAG-3'	258	60
9	5'-ATCCTGCCTAAAATGCTTTTGC-3'	5'-AAAGCAGGAAAAAGTGCTGC-3'	2000	58
19	5'-GCTTGGGCTAGTTTGCTATCTC-3'	5'-TGAGTTGGTGCTATACACGGAG-3'	304	62
21	5'-CCAAAATTGTTCTTCAGTACACC-3'	5'-ATCTGGCAGCACACTGGTATG-3'	258	64

^aGenBank accession nos: U73911–U73915.

cDNA sequences corresponding to 5' and 3' *Pex* exons were shown to be deleted in Gy and Hyp mice, respectively. To determine the intragenic breakpoints, RT-PCR products were hybridised to a mouse genomic DNA panel (Fig. 3). The Gy breakpoint was determined to map between exons 3 and 4. The three *HindIII* fragments corresponding to exons 1–3 are deleted (Fig. 3a). In Hyp mice, six *EcoRI* fragments corresponding to exons 16–22 were found to be deleted (Fig. 3b). The three *EcoRI* bands that correspond to exons 12–15 were present. Thus, the deletion breakpoint in Hyp lies between exons 15 and 16.

The *Pex* deletions in Hyp and Gy mice were confirmed by hybridising genomic fragments containing exons 3 and 21 and by amplifying genomic DNA with intronic primers flanking exons 3, 19 and 21 (Table 1). The 2.9 kb *EcoRI*–*HindIII* fragment from P1 clone 703H24279 containing exon 3 is absent in Gy mice and no product could be amplified from Gy genomic DNA using intronic primers flanking that exon. In Hyp mice, the 1.3 kb *EcoRI* fragment from P1 clone 703B06300 containing exon 21 is absent. Furthermore, in Hyp mice, it was not possible to amplify exons 19 and 21 with intronic primers.

We have mapped the spermine synthase gene distal to *PEX*. Analysis of human cosmids in this region indicates the distance between spermine synthase and *PEX* to be ~40 kb. The ORF of the human spermine synthase gene shows the same orientation as *PEX*. The genomic organisation of the human spermine synthase has been determined independently (GenBank accession no. U53331). The homologous mouse sequence was isolated from an embryonic cDNA library and is highly conserved (GenBank accession no. Y09419). So far we have no evidence that sequences at the 3' end of the spermine synthase gene are deleted in Gy. Sequences of exon 2 and of the 3'-untranslated region (UTR) of the spermine synthase gene can be amplified from Gy genomic DNA (see Materials and Methods).

DISCUSSION

We have determined the cDNA sequence of the mouse homolog of *PEX* by isolating genomic clones, subcloning and sequencing positive fragments and performing RT-PCRs between the isolated exons. The *PEX* gene appears to be weakly or transiently expressed in most tissues, as shown by Northern blot results (1) and the rarity of clones found in cDNA libraries. We identified only a single cDNA clone from one million clones screened of a mouse spleen library.

Determination of the genomic structure of five mouse exons and hybridisation of cDNA fragments of various length provide evidence that the genomic organisation of the mouse gene is comparable with the human gene, which covers in total ~220 kb and consists of 22 exons (F. Francis, in preparation). The mouse

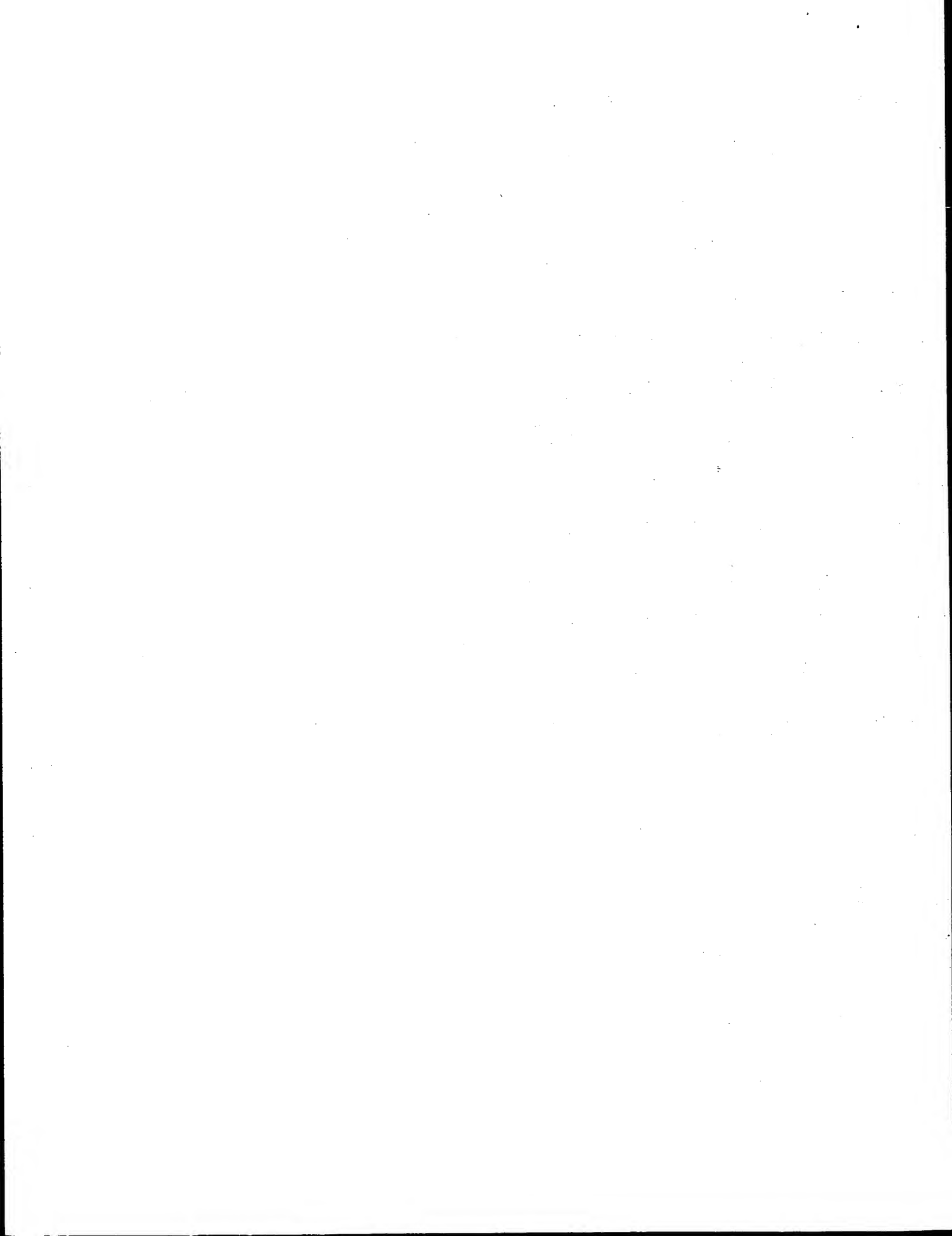
P1 clone, 703B06300, has an insert size of ~20 kb and contains the exons 19–21. The mouse P1 clone, 703H24279, which was examined most extensively, has an insert size of ~70 kb and contains the first nine *Pex* exons. The corresponding human exons are encompassed in a genomic region of ~67 kb.

The derived *Pex* cDNA sequence shows a high homology to its human homolog (Fig. 2). This high interspecies sequence conservation is a known characteristic of the neutral endopeptidase family (30). Both *Pex* and its human homolog contain only the 10 cysteines which are conserved throughout the endopeptidase family. They lack the additional cysteine in rat ECE-1 which is known to be involved in homodimerisation (31). The determination of the tertiary structure awaits further studies. To date, the three-dimensional structure of members of the M4, M10 and M12 family of metalloproteases have been determined, but not that of any of the members of the neutral endopeptidase family (25).

Recently, the sequence of *Pex* has also been determined by Du *et al.* (21). No mutation was reported in Hyp mice using a RT-PCR approach. However, no Southern blotting experiments have been performed. The cDNA sequence is identical to the one described here, with the exception of a silent nucleotide substitution at position 2235 from T to C. In the 5'-UTR we found three additional nucleotides at position -149, -158 and -159.

The deletions we have identified in both the Hyp and Gy mice were found to be non-overlapping. While Gy bears a deletion of the first three exons of *Pex* including upstream sequences, in Hyp mice the last seven exons and downstream sequences of unknown size are deleted. Both deletions are predicted to inactivate the mouse gene fully. The occurrence of a deletion in Gy mice is in accordance with the high frequency of deletions observed in irradiation experiments. The observation of a deletion in the spontaneous Hyp mutation is mirrored by the finding of intragenic deletions in humans which occur at a frequency of ~20%. According to the human genomic sequence, the distance between the Hyp and Gy deletion measures minimally 100 kb. The reported recombination between the Hyp and Gy locus (8) must have occurred within this region. Similarly, a translocation breakpoint within the human gene has been identified: a female with hypophosphatemic rickets has been found to have triple X and a balanced translocation between Xp22 and chromosome 6p. The breakpoint on the X chromosome is located within a 14 kb *EcoRI* fragment which contains exons 17–20 (data not shown).

The Hyp mouse provides a mouse model for X-linked hypophosphatemic rickets in man. Like the mutations in man, the Hyp mutation leads to a non-functional protein. The Hyp phenotype resembles the symptoms observed in the human disease, which are rachitic bone disease and hypophosphatemia arising from impaired renal reabsorption of filtered phosphate.



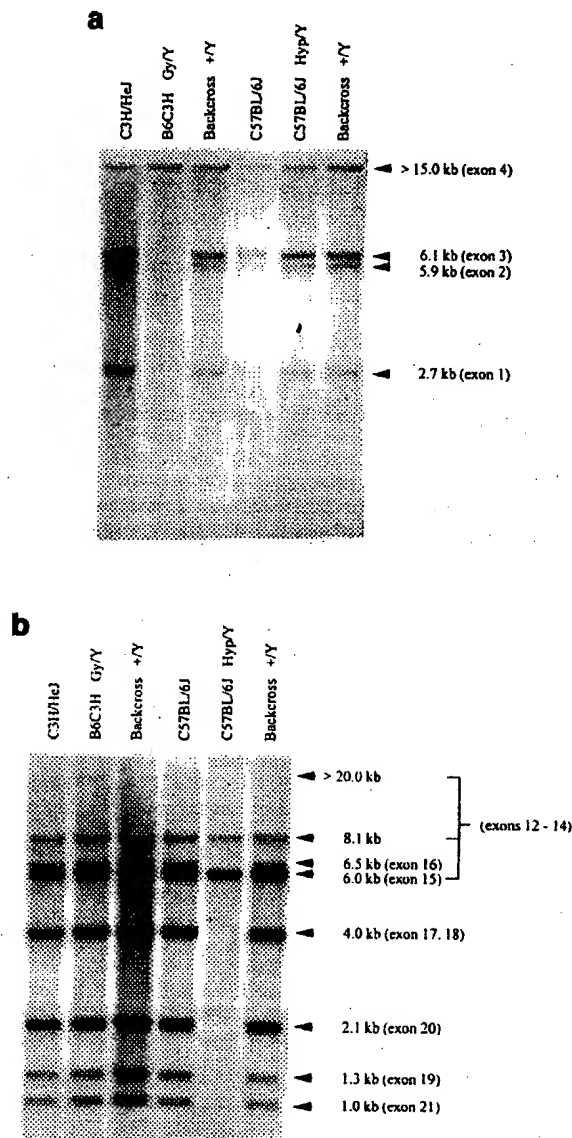


Figure 3. Southern blot analysis of genomic DNA derived from Gy and Hyp mice, background strains and backcrosses. (a) Determination of the intragenic deletion breakpoint in Gy. Hybridisation of a RT-PCR product corresponding to human exons 1–4 to *HindIII* digests of genomic DNA. The >15 kb band corresponding to human exon 4 is present in all lanes. The 2.7, 5.9 and 6.1 kb bands corresponding to human exons 1, 2 and 3, respectively are absent in Gy mice but present in control and Hyp mice. (b) Determination of the intragenic deletion breakpoint in Hyp. Hybridisation of a RT-PCR product corresponding to human exons 12–21 to *EcoRI* digests of genomic DNA. The 6.5, 4.0, 1.3, 2.1 and 1.0 kb bands corresponding to human exon 16–21 are absent in Hyp mice but present in control and Gy mice. In addition, 6, 8.1 and >20 kb bands which correspond to human exons 12–15 are present in all lanes.

In contrast to Hyp, the Gy phenotype is more complex. In addition to the changes in phosphate metabolism, male Gy mice exhibit hyperactivity, circling behavior, inner ear abnormalities and sterility. The prevalence of circling behavior increases with age. On the C57BL/6J background male Gy mice do not survive, whereas on the B6C3H viability is decreased compared with normals (20). The symptoms of the Gy phenotype could be

explained by a contiguous gene deletion syndrome which involves at least one additional gene to *Pex*. So far, we have no evidence that the spermine synthase gene is deleted in Gy mice. Both 3' and 5' sequences can be amplified by PCR, but a gross rearrangement cannot be excluded. The existence of further genes in between spermine synthase and *Pex* also cannot be excluded. Interestingly, a form of non-syndromic deafness (DFN6) recently has been linked to Xp22 within a candidate region which encompasses the *PEX* gene (32).

With two mouse models for hypophosphatemic rickets available, functional studies are facilitated. Experiments that profit from the existence of mouse models include expression studies, the search for modifier genes, the testing of hypotheses regarding the pathophysiology of the disease and the testing of novel therapeutic approaches.

MATERIALS AND METHODS

Mice

DNA from B6C3H *a/a*+/Y and *A/a*–Gy/Y and C57BL/6J +/Y and Hyp/Y mice were obtained from the Jackson laboratory.

cDNA library screening

Approximately 1 000 000 clones were plated out from an oligo(dT) mouse spleen (Clontech ML1018a) and a mouse lymphocyte library (Clontech ML1032a). The libraries were screened by filter hybridisation using human *PEX* cDNAs C2H20, C110E and pac1M21 (1) as probes. A single positive phage clone from the spleen library was purified by rescreeing.

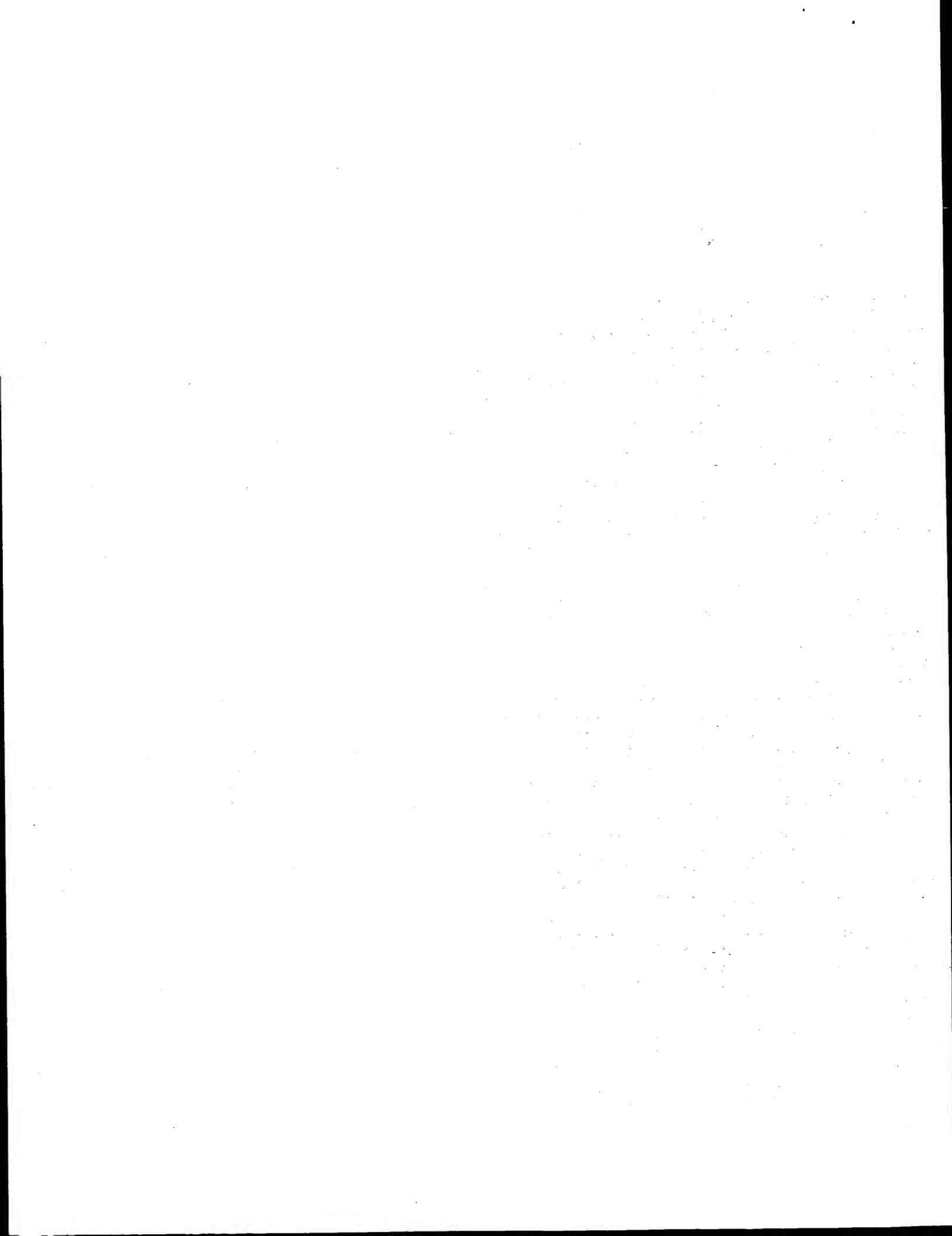
A human cDNA clone containing the 3'-UTR of spermine synthase was isolated from a selected cDNA library (1). This cDNA was used to identify clones in mouse 9 day and 12 day embryonic cDNA libraries (B. Herrmann, unpublished).

Identification of P1 clones and subcloning

P1s were identified by screening human *PEX* cDNAs C110E and P220L (1) against gridded mouse P1 library filters. This P1 library was prepared using *MhoI* partially digested DNA obtained from C57BL/6 female mice spleen (F. Francis, unpublished). The cloning vector was pAd10sacBII and the host strain NS3145 (33). Clones (120 000) were picked robotically into microtiter dishes, and arrayed at high densities on filter membranes. Hybridisations with human cDNA clones were performed at low stringency in a 30% formamide hybridisation buffer at 42°C. Washes were performed in 3× SSC, 0.1% SDS for 2× 20 min at 20°C, followed by 2× 20 min at 65°C. P1s were prepared as described (34). P1 clones were digested with *EcoRI*, *EcoRI*–*HindIII*, *HindIII* and *PstI* (Boehringer Mannheim) and hybridised with human *PEX* cDNAs C2H20, C110E, C116 and P220L. Positive fragments were subcloned into Bluescript II SK +/– (Stratagene) and pUC19 (Gibco BRL) plasmid vectors and sequenced using SK and pUC19 primers. From this sequence, primers (Table 1) were designed to amplify the mouse exons.

RT-PCR assays and 5' and 3' RACE

For RT-PCR, poly(A)⁺ RNA was prepared from 12.5 day mouse embryo (brain) and adult mice (muscle and bone) by extraction with guanidine isothiocyanate and polystyrene latex particles with covalently linked dT30 oligonucleotides (Qiagen). First



strand synthesis was carried out using *Pex*-specific primers from the 5' and 3' end and reverse transcriptase (Pharmacia). As PCR template, 1–10 ng of first-strand cDNA was used for nested PCR reactions. Cycling profiles included an initial denaturation step (94°C for 5 min) followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final extension step (72°C for 5 min). The amplification product was used for direct sequencing.

RACE were performed with the ready Marathon cDNA Amplification Kit from 17 day mouse embryo (Clontech). Nested 5' RACE was performed using the adaptor-specific primers AP1 and AP2, and *Pex*-specific 5' end primers (A1MR: 5'-TCGGCTGACTGATTTCTCCAG-3', E2MR1: 5'-TCCAGC-CATCACAAGCAAACC-3'). Nested 3' RACE was performed using AP1 and AP2, and *Pex*-specific 3' end primers (B11MF0: 5'-TGAAAGGGAAGAGGACCCTG-3', B11MF1: 5'-ATTGCT-GATAATGGGGTCTG-3'). The PCRs were carried out essentially as recommended by the manufacturer. Products were gel purified and sequenced.

Sequencing

PCR-amplified cDNAs, library cDNA clone and genomic fragments cloned into plasmid vectors were purified by PCR purification, gel extraction and plasmid preparation kits (Qiagen). Cycle sequencing was performed using a Taq DyeDeoxy Terminator Cycle sequencing kit (ABI). The sequences were determined with an Applied Biosystems 377 automated sequencer.

Mutation analysis

For Southern blot analysis, genomic DNA (~5 µg) from mouse inbred strains (C3H/HeJ and C57BL/6J), mouse mutant strains (B6C3H Gy/Y and C57BL/6J Hyp/Y) and backcross strains were digested with *Eco*RI and *Hind*III. The digested DNAs were electrophoresed on 0.7 and 1% agarose gels, and blotted to nylon membranes (Hybond-N⁺, Amersham) by alkaline transfer. Hybridisations were generally performed in a hybridisation buffer containing 1.5× SSPE, 1% SDS and 10% dextran sulfate at 65°C. Probes were labeled by random hexamer priming. Washing was done under stringent conditions (0.1× SSC, 0.1% SDS at 65°C for 15 min).

Primers designed from genomic sequence were used for DNA amplification of *Pex* exons (Table 1). After an initial denaturation for 5 min at 94°C, denaturation was at 94°C for 40 s, annealing at the exon-specific temperature for 40 s, and 40 s extension at 72°C for 30 cycles followed by a final extension for 5 min at 72°C. Reactions (50 µl) contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.01% (w/v) gelatine, 160 µM of each dNTP, 0.6 µM of each primer, 0.5 U *Taq* DNA polymerase (USB), and 100 ng of genomic mouse DNA.

For amplification of spermine synthase, we used primers designed from the cDNA sequence (GenBank accession no. Y09419). Amplification of genomic DNA was performed with primers of exon 2 (SSmF1: 5'-CCAAAGCTGATGGTGAGG-3', SpSyR3: 5'-CATTCCTGTTCTGTAAGTTGC-3') and of the 3'-UTR (SpSyF1: 5'-TTTTACTGTTTGAAGAAAGC-3', SpSyR1: 5'-CTAAGTCAATTGGGGGTGAG-3').

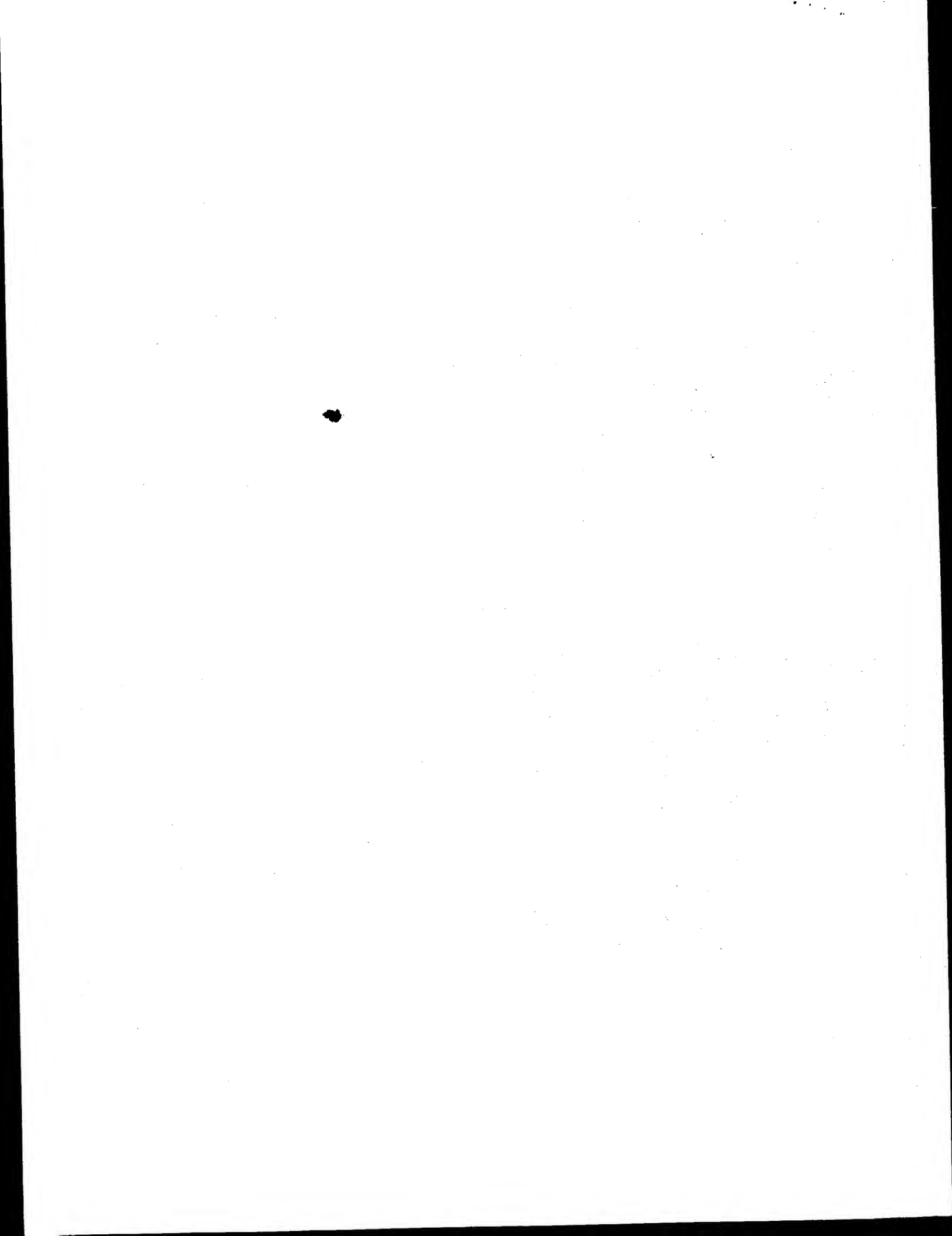
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CORPORATE SOURCE: M.J. Econs, Dept. of Medicine, Indiana University Medical Center, 975 W. Walnut St., Indianapolis, IN 46202, United States

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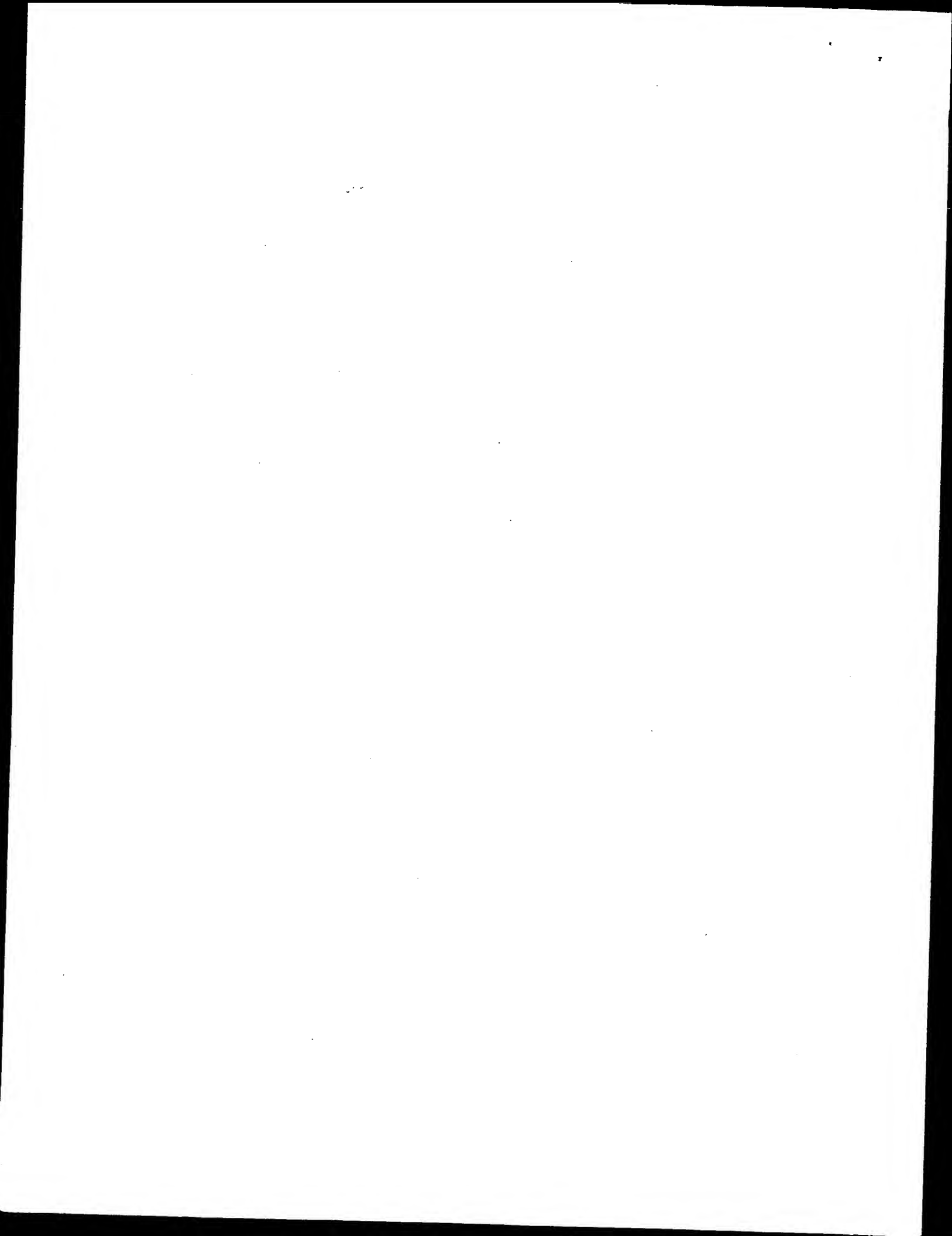
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NEW PERSPECTIVES ON THE BIOLOGY AND TREATMENT OF X-LINKED HYPOPHOSPHATEMIC RICKETS

Thomas O. Carpenter, MD

NOMENCLATURE

Initial descriptions of hypophosphatemic rickets emphasized the poor clinical response to vitamin D, rather than the distinctive finding of marked hypophosphatemia. Such early descriptions became the basis for nomenclature of the rachitic disorders; so the name *vitamin D-resistant rickets* was widely used before the recognition that many cases were secondary to renal phosphate wasting. The name, *vitamin D-resistant rickets*, was well-entrenched by the time a more physiologic description was suggested. To add to the confusion, a target organ form of vitamin D resistance is now well characterized and is caused by mutations in the $1,25(\text{OH})_2\text{D}_3$ receptor gene, analogous to other hormone-resistant states. This disorder of the $1,25(\text{OH})_2\text{D}$ receptor, previously named *vitamin D-dependent rickets, type 2* is increasingly referred to as *hereditary resistance to vitamin D*. Thus the currently preferred terminology for the hypophosphatemic disorder, *X-linked hypophosphatemic rickets* (XLH) (or in some texts, *familial hypophosphatemic rickets*) distinguishes the condition from true biochemical resistance to vitamin D and refers to the primary mineral disturbance.

A variety of hypophosphatemic rachitic disorders exist. The more frequently encountered disorders are secondary to inappropriate urinary excretion of phosphate. We refer to the larger group of renal phosphate wasting disorders as

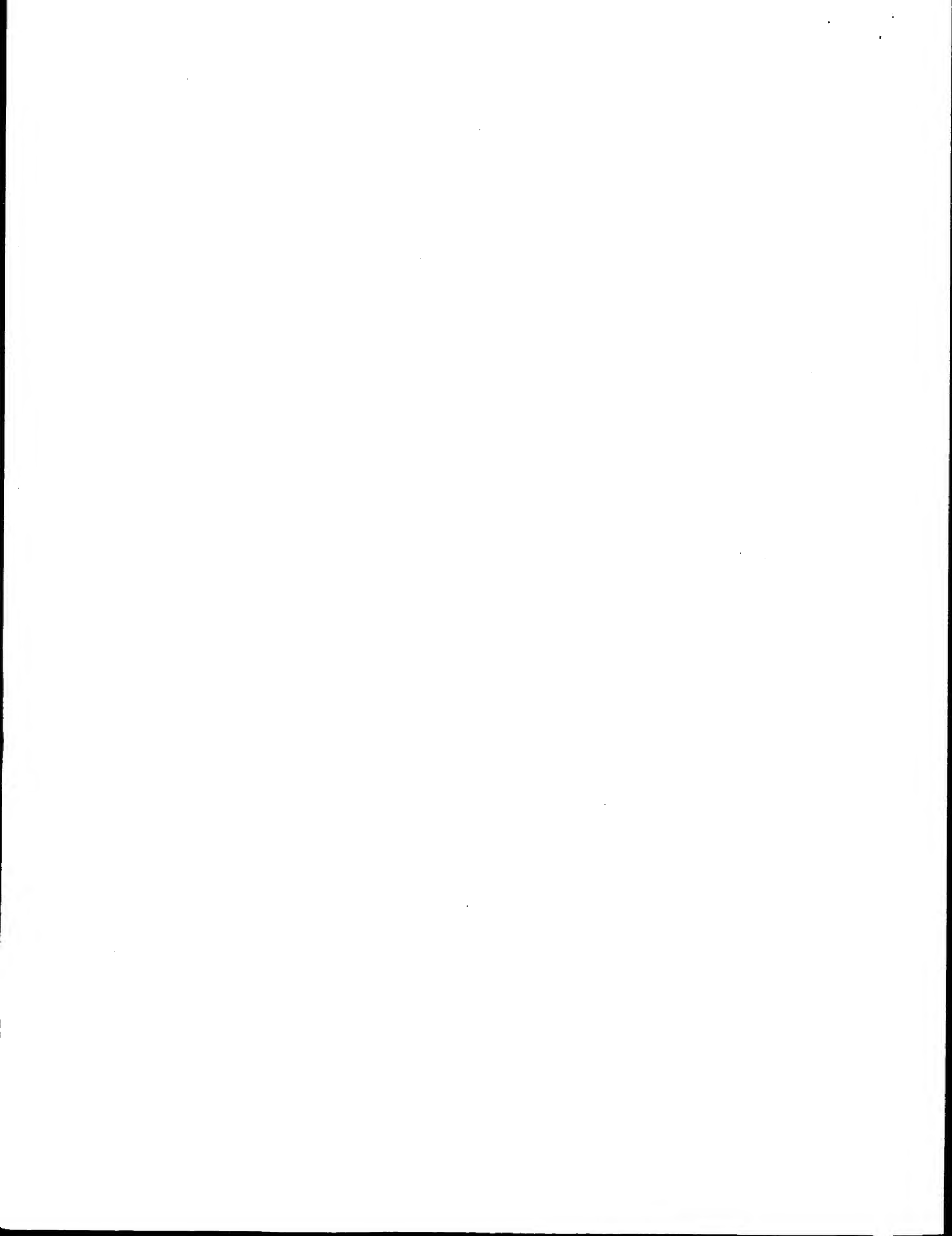
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443



primary hypophosphatemic rickets, with the understanding that in children, the most commonly identified cause in this group is XLH. XLH is transmitted in X-linked dominant fashion, but other patterns of inheritance have been described. In most children sporadic occurrence of primary hypophosphatemic rickets is probably caused by spontaneous mutations in the affected gene on the X chromosome, and subsequent generations transmit the disorder in an X-linked dominant fashion.⁷¹ Incidence has been estimated between 1 in 10,000 and 1 in 100,000, and 1 per 20,000 population seems to be the most widely quoted figure.⁷⁴

CLINICAL DESCRIPTION OF XLH

Presentation and Diagnosis

In the absence of pertinent family history, the typical presentation of a child with XLH occurs in the second or third year of life. There is often an observation by a family member who intermittently sees the child (often grandparents) that the legs are bowed. The finding is often attributed to physiologic bowing of childhood, and the appearance of the legs is observed over the course of 6 to 12 months. In children with rickets, there is a continued progression of bow deformity in the lower extremities that does not resolve with growth. Pediatric endocrinologists are often asked, "At what point is physiologic bowing of childhood no longer a sufficient explanation for a given child's bow-legged appearance?" The author recommends that progression of the bow defect over a 6-month period in a child more than 18 months of age requires further radiographic and biochemical investigation. In cooperative children, the degree of bow can be ascertained by measurement of the interpopliteal distance with a small ruler. The child can be standing or laying prone with the ankles placed together. A convenient anatomic marker is the dimple of the popliteal fossa (Fig. 1). The distance between the right and left dimples should not increase by more than 1.0 cm over a 6-month period in normal children. Others prefer to measure the space between the medial tibial condyles.

In the absence of family history, clinical manifestations compel the diagnostic investigation. An obvious bow defect, or progression of a mild bow that is originally believed to represent physiologic bowing of childhood, should be cause for simple radiographs of the knees. Lower extremities are almost always more extensively involved than upper extremities in patients with XLH, and further radiographic examination usually is unnecessary if this diagnosis is made. Interestingly, this differential involvement of the long bones is not typical of calciopenic rickets, such as that seen with vitamin D deficiency, in which radiographic manifestations at the distal ulna may be pronounced. Characteristic rachitic deformities of the distal femur generally are present in patients with XLH by 1 year of age, although a considerable variation in severity is encountered. In the sporadic case, an exhaustive search for the occurrence of small tumors is also important to exclude the diagnosis of oncogenic hypophosphatemic osteomalacia.

An initial biochemical investigation should include determination of serum calcium, serum phosphorus, and serum alkaline phosphatase activity. Care must be taken with respect to the interpretation of serum phosphorus values. Many laboratories provide an adult reference range (which is much lower for serum phosphorus than in children) with their reports, resulting in inappropriate interpretation of hypophosphatemia in children as normal. The author has not uncommonly encountered misdiagnoses on this basis. A low serum phosphorus

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a children, the transmitted in X-linked hypophosphatemic rickets is on the X chromosome. The prevalence is 1 in 10,000 and 1 in 100,000 in quoted figure.⁷⁴

ation of a child (an observation of parents) that of bowing of the tibiae of 6 to 12 cm of bow deformity. Pediatric bowing of the tibiae is a bow-legged deformity over the knees. Further, the degree of bowing distance with the ankles placed together (Fig. 1) is measured by more than 1 cm to measure

all the diagnosed bow that is normal, should be almost always with XLH, and the diagnosis is not typical of rickets, in which characteristic bowing of the tibiae with bowing of the tibiae is encountered of small bowing of the tibiae hypophosphatemic

ation of serum phosphorus. Care must be taken for serum phosphorus. Inappropriate serum phosphorus has not been reported

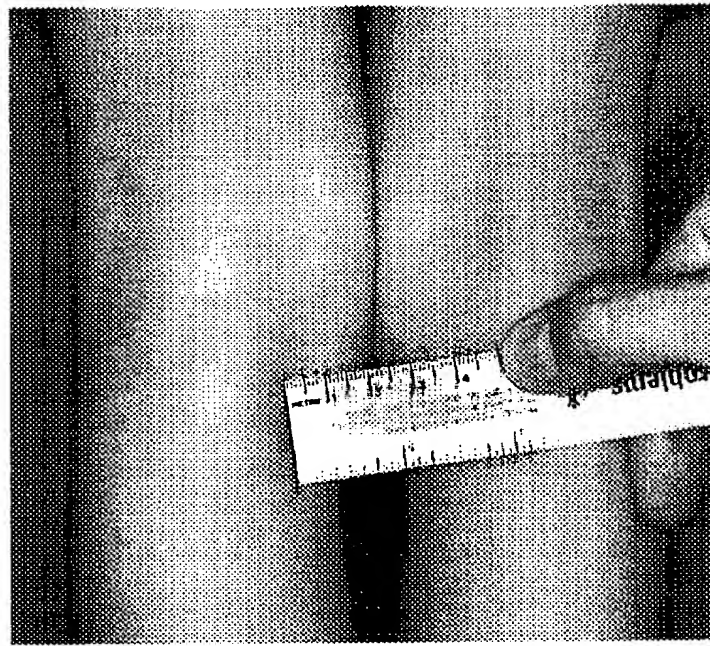


Figure 1. The space between the left and right popliteal dimples can be readily measured with a small ruler while the child is standing with the ankles touching; the interpopliteal distance in this normal girl is 4.5 cm. The author finds this measurement is more easily obtained than an intercondylar distance.

level in combination with elevated serum alkaline phosphatase activity and normal serum calcium is suggestive of primary hypophosphatemic rickets. Diagnosis depends on an accurate assessment of renal phosphate handling. A convenient approach is to obtain a 2-hour urine collection after a 4-hour fast for determination of creatinine, phosphorus, and calcium levels. Tubular reabsorption of phosphate (TRP) is calculated from urine and simultaneous blood values (usually sampled at the midpoint of the timed urine collection) as follows:

$$\%TRP = 1 - \frac{(\text{Urine P} \times \text{Serum Creatinine})}{(\text{Serum P} \times \text{Urine Creatinine})} \times 100$$

with units for phosphorus and creatinine expressed in mg/dL. The normal TRP in children is 85% to 100%. It is best to interpret this number in view of the filtered phosphate, as deduced from the serum P value. A nomogram (Fig. 2) can be used to determine the TMP/GFR, or renal tubular threshold maximum for phosphate, as expressed per glomerular filtration rate.¹⁰⁶ Normal values for TMP/GFR are age-related, approximating the normal ranges of serum phosphorus for age. A low TMP/GFR in the setting of a low serum P value in this setting documents the inappropriate renal phosphate losses characteristic of primary hypophosphatemic rickets. Finally, alkaline phosphatase activity usually is elevated in children with XLH, but usually less than the tenfold to 15-fold elevations often seen in patients with vitamin D deficiency or hereditary resistance to vitamin D. Adults with XLH may have normal serum alkaline phosphatase activity values despite impressive osteomalacia; thus monitoring of

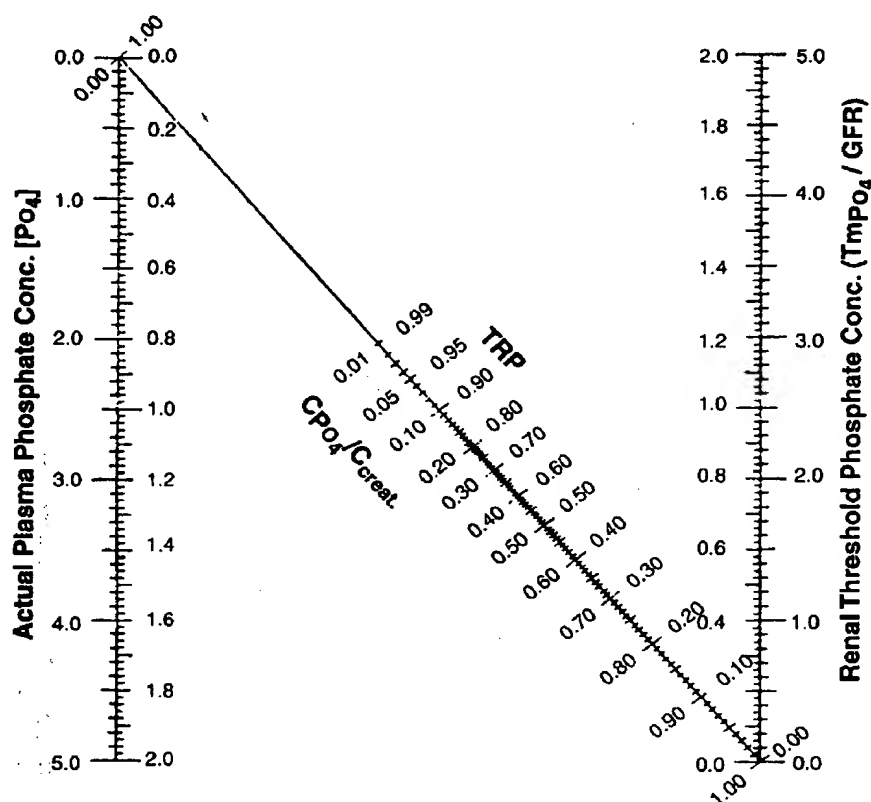


Figure 2. Nomogram for determination of the renal tubular threshold maximum for phosphate. The concentration of plasma phosphate (*left axis*) is plotted to intersect with the calculated tubular reabsorption of phosphate (TRP) (*along the central diagonal*), thereby intersecting with the renal tubular threshold maximum for phosphate (TMP) on the right axis (mg/dL units are represented by the exterior scales, and mmol/L units are represented on the interior scales of each vertical axis). (From Walton RJ, Bijvoet OL: Nomogram for derivation of renal threshold phosphate concentration. *Lancet* ii:309, © 1957 The Lancet Ltd.; with permission.)

serum alkaline phosphatase is not a particularly sensitive indicator of response to therapy, particularly in adults.

A secondary level of laboratory investigation is useful to confirm the diagnosis. In addition to the earlier-mentioned tests, the author recommends measurement of parathyroid hormone (PTH) levels and the circulating vitamin D metabolites, 25-OHD and 1,25(OH)₂D, before the onset of therapy. Determination of urinary calcium excretion (which can be performed on a 24-hour collection or a briefer timed collection if sufficient urinary volume is collected) is important to distinguish the disease from hypercalciuric variants. Characteristically, 25-OHD levels are normal, distinguishing the disorder from vitamin D deficiency, and circulating 1,25(OH)₂D levels are normal, but inappropriately so given the ambient hypophosphatemia, which normally serves to increase circulating levels of this metabolite. Circulating PTH levels may be normal or elevated, even

before the onset of therapy, but usually less than 100 pg/mL in the absence of thyroidism is this article.

A discussion of the clinical features mentioned by the colleagues³² and members of the task force. Significant bone loss was present in all patients who were aware of

Skeletal Phenomena

Rickets, a disorder of the developing skeleton, is affected than in adults. In severe forms, the deformities are apparent at the onset of life, with fraying of the epiphyses often manifesting in the patient may have anterior bowing of the legs. The asymmetry of the growth plate may result in a lateral deviation of the extremity. The asymmetry of the growth plate may result in a lateral deviation of the extremity.

Figure 3. Radiograph showing the severity of the disease. The epiphyses are wider and more

before the onset of therapy in XLH; however, the magnitude of elevation is usually less than in calciopenic forms of rickets. The development of hyperparathyroidism is a common complication of therapy and is addressed later in this article.

A discussion of the clinical presentation of XLH is incomplete without mention of the important survey performed several years ago by Econs and colleagues.³² This study revealed a remarkable lack of awareness among 234 members of three large kindreds about the disease or their diagnostic status. Significant bone, joint, and back pain; dental abscesses; weakness; or hearing loss was present among the 57 affected individuals, but less than 25% of them were aware of their diagnosis, and only one was receiving any specific therapy.

Skeletal Phenotype

Rickets, manifest as a bow or knock-knee deformity, is the most prominent skeletal feature of children with XLH. Lower extremities are more severely affected than the arms, ribs, or pelvis, sites that are often prominently affected in severe forms of calciopenic rickets. Features characteristic of rickets are most apparent at the ends of long bones, radiographically manifest as flared metaphyses with frayed borders, which may develop a cupped configuration. The femora often manifest a bow defect in the anterior-posterior and lateral directions; the patient may assume an exaggerated lordotic posture to compensate for the anterior bow. As bowing progresses in later childhood, an asymmetric appearance of the growth plates may become evident (Fig. 3), such that medial and lateral aspects of the growth plate manifest a differing severity of the abnormality. The asymmetry is attributed to altered weightbearing forces through the physis resulting from the bow. Asymmetric premature closure of the growth plate may occur, which can further exaggerate the bow defect, because one side of the extremity continues to grow, and the other side cannot.³³ With fusion, growth plate abnormalities per se are no longer apparent, although if deformities

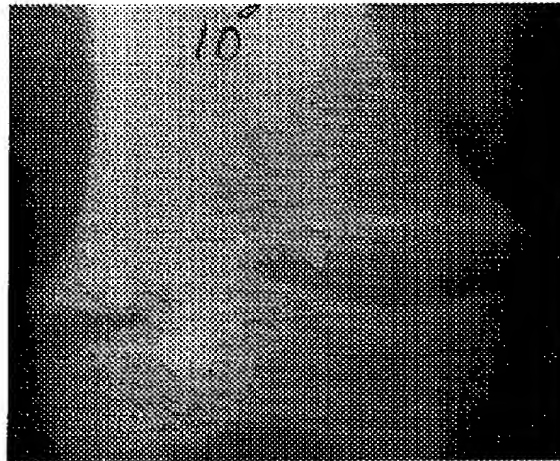


Figure 3. Radiograph of the distal femur in a child with XLH. Note the asymmetry of the severity of the rachitic lesion at the growth plate, with the medial physis being considerably wider and more frayed than the lateral physis.

are severe in childhood, abnormal configuration of epiphyses may persist into adulthood. Patients may develop pseudofractures, but this development is more common in the adult population.

The characteristic microscopic finding in biopsy specimens of both trabecular and cortical bone is osteomalacia.^{51, 52} There is an increase in histomorphometric quantitation of unmineralized bone matrix or osteoid; however, total bone volume may be normal or elevated.^{76, 94} Cellularity of bone is usually reduced; however, in patients that manifest hyperparathyroidism, increased cellular parameters of bone turnover (e.g., cell number or osteoblastic or osteoclastic surface) may be present.⁹⁴ Dynamic histomorphometry shows a lag in mineralization time.^{51, 52, 76, 94}

Studies of bone density in patients with XLH must be interpreted carefully with respect to methodology used.^{48, 76, 82} Studies using absorptiometry techniques suggest that bone mineral density of the spine is normal or increased in patients with XLH.^{76, 82} This finding may, in part, be related to the tendency of affected individuals to develop extra ossicles, osteophytes, and spinal stenosis. Computed tomographic determination of cortical bone density is low in patients with XLH, despite increased cortical area.⁴⁸ These findings are entirely consistent with histologic findings of increased total bone volume, but with low mineral content per unit of total bone.

Craniosynostosis may occur in childhood but is relatively uncommon. Affected children more commonly manifest a scaphocephalic configuration of the skull. Optic canal stenosis has been reported.¹² Chiari I malformation (inferior displacement of the cerebellar tonsils below the foramen magnum) has recently been described in 7 of 16 patients with XLH and is believed to be related to severe calvarial osteomalacia and thickening.¹¹ A small posterior fossa results and may not allow sufficient intracranial space for normal positioning of the cerebellum. Symptomatology explained by such a defect occurred in only one individual in this series. It has been suggested that patients with XLH who develop severe headaches, nausea, or other changes of increased intracranial pressure be examined for this abnormality. Surgical decompression has been suggested if severe symptoms develop, but the outcomes of this approach are not clear, and such management remains controversial.⁶

Many patients develop other skeletal problems in the third and fourth decades of life. Vertebral abnormalities are not uncommon, with thickening of spinous processes, fusion, thickening of facet joints, and spinal canal stenosis.⁷⁵ In one unfortunate man with XLH who contracted epiglottitis, routine endotracheal intubation proved impossible because extension of the neck was extremely limited because of skeletal changes. Calcification of tendons and ligaments is also a common manifestation of the disease, appearing at various ages, but is unrelated to therapy.⁷⁰ A recent report describes a 49-year-old woman presenting with diffuse enthesopathy, including bony projections of the hands and feet, who was found to be affected with XLH and who had no history of therapeutic intervention.¹⁸

Growth

Significant concerns in the childhood management of XLH are short stature and the outcome of final height. The severity of growth impairment is variable but primarily relates to poor lower-extremity growth. Height outcomes of medical intervention are discussed in the section on therapy.

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Teeth

Dental abscesses are frequently encountered in children and adults with XLH.⁵⁴ This problem is related to the abnormal structure of the teeth in patients with XLH.¹ Enlargement of the central pulp chambers of teeth and elongation of pulp horns to the point of extension to the dentoenamel junction are characteristic. Enlargement of the pulp chamber is associated with irregularities in interglobular dentin. Phosphate content of dentin is low and dentine calcospheres coalesce poorly, resulting in undermineralization. Expansion of the pulp compartment results in a diminished barrier to the exterior of the teeth, such that residua from oral fluids more readily pass through the outer enamel layer to the pulp, initiating abscess formation, even in the absence of caries. The occurrence of one abscess in an individual with XLH suggests that more will occur.⁵⁴ Standard management via pulpectomy and root canal filling may not be successful.⁷² Full crown coverage may be a helpful prophylactic measure. Other suggestions have included composite coatings; however, the use of sealants has been contraindicated because acid material may leak through cracks in enamel to the pulp chamber. Evaluation by a dentist with expertise in managing the dental problems characteristic of patients with XLH is recommended in all patients with XLH in whom a single dental abscess develops.

Male patients have been shown to have more extensive dental disease than female patients, despite comparable levels of circulating phosphate.^{54, 92} This finding suggests that the pathophysiology of the dental disease may not reflect ambient phosphate concentrations but occurs because of an independent abnormality expressed in mineralizing tissue. The male-female difference in severity may reflect either hormonal influences on the expression of the defect in mineralized tissue, or a gene dose effect, because male patients carry a single abnormal copy of the pertinent gene, whereas female patients, with two X chromosomes, are heterozygous for the defect. A positive effect of medical therapy on dental health has not been established.⁹¹

Renal Abnormalities

The renal tubule does not appropriately reabsorb filtered phosphate, resulting in excessive urinary excretion of phosphate. Therefore, the steady state circulating phosphate concentrations are low. For many years, the compromised mineralization of bone has been attributed to this resultant hypophosphatemia. Renal tubular transport abnormalities generally are limited to phosphate, although a modest defect in glucose transport has been described in some families with the disease.⁷⁴ Renal tubular acidosis has been described as a concomitant renal abnormality, but this defect seems to correct with phosphate supplementation.⁵⁷ Bicarbonaturia may occur secondary to concomitant hyperparathyroidism. One study suggests that renal tubular acidosis may increase the risk for nephrocalcinosis; however, no assessment of PTH status was performed.⁹⁰ Nephrocalcinosis is discussed in detail in the section on complications.

Abnormalities in the metabolism of vitamin D are characteristic of patients with XLH. The rate-limiting step in the synthesis of 1,25(OH)₂D occurs in renal tubular mitochondria. 1,25(OH)₂D levels are normal in patients with XLH; however, this finding is inappropriate in view of the low circulating-phosphate level. PTH or a low phosphate diet, normal stimuli for 1,25(OH)₂D production, results in blunted responses of circulating 1,25(OH)₂D when administered to individuals with XLH.⁷⁴ Other abnormalities in vitamin D metabolism, particu-

larly increased 24-hydroxylase activity, have been demonstrated in murine models of XLH.⁶⁰

Parathyroid Hormone

Albright's original report of a patient with primary hypophosphatemic rickets included a histologic description of hyperplastic parathyroid glands.² Other investigators have recorded the occurrence of secondary and tertiary hyperparathyroidism in XLH in association with chronic phosphate therapy.^{16, 36, 81} Histologic findings are consistent with diffuse hyperplasia of the parathyroid glands. Moderate elevations in circulating PTH levels also occur in untreated adult patients and in some patients before the onset of any therapy.^{16, 45} Elevations in circulating PTH levels are more pronounced during nocturnal sampling. These findings suggest that the parathyroids in XLH have an inherent propensity to become hyperplastic.²⁴ PTH levels should be monitored carefully during therapy so that the development of hyperparathyroidism does not complicate efforts to maintain serum phosphorus levels or compromise the skeleton.

Other Manifestations of Disease

The cardiovascular system has been examined in patients with XLH. Early studies reported normal electrocardiograms and left ventricular ejection fractions in this group of patients¹⁰³; however, more recent work has demonstrated abnormalities in blood pressure response to exercise and mild ventricular hypertrophy.⁶⁴ The findings suggest a defect in the regulation of vascular tone, but no causal mechanism has been identified.

Most children with XLH are predominantly affected by the defect in mineralized tissues (skeletal and dental abnormalities, and stature). They are otherwise generally healthy and free of signs and symptoms of total body phosphate depletion. In contrast with rickets related to vitamin D insufficiency, muscle weakness is not common. In the author's experience, the uncommon XLH patient in whom muscle weakness develops is often extremely hypophosphatemic (serum P levels < 1.5 mg/dL) as a consequence of hyperparathyroidism. This clinical observation suggests that intracellular phosphate pools are reasonably well maintained, despite the ambient hypophosphatemia. In acquired adulthood hypophosphatemic osteomalacia, a separate clinical entity, muscle weakness is more frequently encountered.⁷⁴ Osteoporosis has been described in yet another adult population with mild renal phosphate wasting.⁴⁹

RELATED HYPOPHOSPHATEMIC DISORDERS

Poor dietary phosphorus intake has accounted for hypophosphatemic rickets in infancy, particularly in breast-fed premature infants. Increased mineral demands of late-trimester skeletal growth cannot be met by the relatively low phosphorus content of human milk. The use of breast milk fortifiers has markedly reduced the incidence of this problem; however, hypercalcemia has been a problem with aggressive supplementation in extremely small infants.⁵⁹ Inappropriate ingestion of phosphate-binding antacid preparations, resulting in poor intestinal phosphorus absorption, may result in a similar syndrome. Although

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adults have been the usual group practicing this habit, childhood cases have been reported recently.⁶⁹

Descriptions of renal phosphate wasting and rickets occurring in association with activating G-protein mutations have recently appeared,³³ presumably signalling reduction in renal tubular phosphate transport. Acquired forms of primary hypophosphatemic rickets have been described in African children, with onset between 4 and 7 years of age.⁶⁸ Affected children may demonstrate serum alkaline phosphatase activity in a more elevated range than that typical for patients with XLH. Muscle weakness was described as more prominent in these patients than that seen in patients with XLH. Exogenous agents toxic to the renal tubule (e.g., ifosfamide or heavy metals) may result in generalized renal tubular dysfunction or Fanconi syndrome, in which phosphate wasting may occur.

Other inheritance patterns of primary hypophosphatemic rickets have been described, including autosomal dominant and autosomal recessive forms. A mild form of autosomal dominant hypophosphatemia is termed *hypophosphatemic bone disease* because renal phosphate wasting occurs, but only minimal skeletal changes are present.⁸⁸ The disorder responds reasonably well to therapy with active vitamin D metabolites. In patients with autosomal recessive hereditary hypophosphatemic rickets with hypercalciuria (HHRH), renal tubular phosphate handling is impaired, but the appropriate physiologic response of the vitamin D 1 α -hydroxylase to hypophosphatemia remains intact.^{20, 100} Thus circulating 1,25(OH)₂D levels are elevated, resulting in certain clinical features that differ from XLH, including increased intestinal calcium absorption, hypercalciuria, and osteopenic bone. The disorder responds to oral phosphate supplementation alone. Heterozygotes manifest absorptive hypercalciuria.¹⁰¹

A most interesting hypophosphatemic syndrome is oncogenic hypophosphatemic osteomalacia (OHO), because it is strikingly similar to XLH. In patients with this rare condition, tumors secrete a substance or substances that impair renal phosphorus handling (resulting in low circulating inorganic phosphate [Pi] levels), alter vitamin D metabolism, and result in osteomalacia.²⁶ The disease is generally more refractory to medical therapy than XLH, and the defect in vitamin D metabolism is more severe, often resulting in frankly low circulating levels of 1,25(OH)₂D. The syndrome is cured on tumor removal. The predominant pathologic diagnoses are small benign hemangiopericytomas and ossifying mesenchymal tumors. The striking parallels of oncogenic osteomalacia to XLH raise the possibility that a similar humoral factor is at work in both conditions. Recent reports have shown that a factor with Pi transport inhibitory activity *in vitro* is produced by cultured tumor cells from patients with oncogenic osteomalacia^{83, 107}; the author has been able to confirm this work in his laboratory (patient material provided by P. Backeljauw, MD, Charlotte, NC).

MURINE MODELS OF XLH

Almost 20 years ago, investigators at the Jackson Laboratories (Bar Harbor, ME) reported a mutant strain in mice, *Hyp*, which manifests rickets and osteomalacia, hypophosphatemia, and defective renal tubular reabsorption of Pi.³⁴ These characteristics are transmitted in an X-linked dominant fashion, as in XLH. So *Hyp* mice have become an extremely important tool in which to investigate the pathophysiology of human XLH. Initial characterization of this model centered on the defect in renal tubular Pi reabsorption. An abnormality in Pi transport can be demonstrated at the apical (brush border or luminal) membrane of

proximal tubular epithelium and is characterized by a decreased apparent V_{\max} in a sodium-dependent Pi transport system.⁹⁷ Expression of a *Hyp* sodium-dependent renal phosphate transporter has been demonstrated in *Xenopus* oocytes⁶²; however, this transporter is not encoded on the X chromosome.⁴⁷ The mechanism by which the X-linked mutation results in impaired Pi conservation in the *Hyp* mouse remains uncertain. A host of other abnormalities have been described and are as follows:

- **Altered regulation of the vitamin D metabolic pathway occurs.** *Hyp* mice demonstrate defective regulation of circulating $1,25(\text{OH})_2\text{D}$, as do humans affected with XLH.⁷⁴ *Hyp* mice demonstrate reduced renal 1α -hydroxylase activity after calcium and vitamin D, or Pi deprivation,^{17, 50, 96} and after parathyroid hormone administration,⁶⁶ manipulations that normally stimulate this enzyme complex. Furthermore, 24-hydroxylation (the initial step in catabolism of $1,25(\text{OH})_2\text{D}$) is increased in *Hyp* mice and may contribute to the lower circulating levels in *Hyp*.⁹⁹
- **Decreased intestinal calcium and phosphate absorption** occur in young *Hyp* mice, but normal absorption is present in adult *Hyp* mice. Decreased intestinal levels of the 9-kDa calcium binding protein (calbindin) accompany this finding.¹⁰
- **Elevated rates of gluconeogenesis** occur in bone cells and renal tubular cells in culture and in freshly isolated renal tubules from *Hyp* mice.^{13, 80} The increase in gluconeogenesis cannot be attributed to hypophosphatemia per se, which serves to decrease rates of gluconeogenesis.
- **Increased basal metabolic rate** and increased oxygen and food consumption are found in *Hyp* mice when compared with normals.¹⁰² The basis for these findings is unknown, although circulating thyroid hormone levels are normal. The percentage of cardiac output distributed to liver, bone, muscle, and skin is increased in *Hyp* mice.
- **Circulating PTH** in *Hyp* mice has been variably reported as normal or elevated,⁷⁴ whereas hypophosphatemia, independent of changes in serum calcium, decreases PTH synthesis and parathyroid cell size.^{44, 63} Thus PTH levels in *Hyp* mice are inappropriate for the ambient hypophosphatemia.
- **Bone related abnormalities.**

Mineralization. Data from osteoblast cross-transplantation experiments confirm a role for ambient hypophosphatemia in the development of mineralization abnormalities in *Hyp* but also suggest that a primary abnormality is present in the mutant osteoblast.²⁹⁻³¹ Pi transport is normal in isolated *Hyp* osteoblasts and fibroblasts.^{28, 79}

Osteoblastic proteins, pH. Despite normal $1,25(\text{OH})_2\text{D}_3$ binding and receptors, circulating osteocalcin is markedly increased in *Hyp* mice, and levels decrease in response to $1,25(\text{OH})_2\text{D}_3$.³⁹ *Hyp* osteoblasts express underphosphorylated osteopontin,⁷⁸ which results from nearly absent activity of a Golgi membrane kinase, casein kinase II.⁵ *Hyp* osteoblasts maintain a slightly lower steady state pH than normals.⁸⁰

Thus there is considerable evidence for multiple defects in *Hyp* mice, and the predominant abnormalities have been described in kidney and bone. *Gy* mice are yet another murine model of X-linked hypophosphatemia and have many characteristics comparable to *Hyp* mice.⁹⁸

INTRACELLULAR PHOSPHATE STATUS

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studies demonstrate that the P_i content in extracts of kidneys from normal and *Hyp* mice are equivalent, and greater than that in normal mice deprived of dietary phosphorus.⁹ These data have been confirmed by the author's study using ^{31}P -NMR spectroscopy to investigate P_i content of living mouse kidney in situ,¹⁴ and others.²³ In contrast, P_i content of muscle tissue in humans with XLH has been described as lower than normal, and enhanced with calcitriol and phosphate therapy.²² The finding of normal intracellular renal P_i content in *Hyp* mice raises problems with the hypothesis that the primary site of renal transport interruption in *Hyp* is in the brush border membrane.

EVIDENCE FOR HUMORAL MEDIATION OF PHOSPHATE TRANSPORT

The hypothesis that a circulating factor has a role in the pathophysiology of XLH was suggested when an individual with XLH underwent renal transplantation, yet the abnormality in renal P_i conservation persisted following the procedure.⁶¹ Renal P_i wasting in normal mice following parabiotic union with *Hyp* mice has been demonstrated.^{55, 56} Furthermore, cross-transplantation experiments have shown that renal P_i wasting persists in *Hyp* mice after their native kidneys are removed and replaced with a normal kidney, whereas normal mice transplanted with *Hyp* kidney do not demonstrate P_i wasting.⁶⁵ The possibility that such a circulating factor is derived from kidney is unlikely, because the transplanted mutant kidney fails to reproduce the syndrome in a normal mouse. Finally, the occurrence of OHO is an example of a humorally mediated, paraneoplastic form of hypophosphatemic rickets that bears striking resemblance to XLH. In sum, these observations support the notion that a humoral factor is important in the pathogenesis of the disease.

GENETIC ADVANCES

Early investigations fostered the speculation that the genetic defect in XLH directly involves a phosphate transport protein or a phosphate-regulating humoral factor. Thus the recent finding that mutations in the novel *PEX* (phosphate regulating gene with homologies to endopeptidases, on the X chromosome) gene were discovered in patients with XLH⁴² was an interesting surprise. The *PEX*-encoded protein is a member of a larger family of endopeptidases with homology to the neutral endopeptidase and endothelin-1 activase, which degrade or activate peptide hormones. These endopeptidases are characterized by the presence of a single membrane spanning domain and a relatively large extracellular region that contains a zinc-binding motif (Fig. 4). The *PEX* protein is expressed in very low abundance, and tissues of physiologic expression have not yet been identified in humans. A murine *Pex* gene is expressed in relative abundance in bone, but is underexpressed in bone from *Hyp* mice.²⁷ Interestingly, there does not seem to be a mutation in the coding region of the *Pex* gene in *Hyp* mice.

Therefore, it seems likely that the *PEX* product is involved in the processing of a factor involved in P_i homeostasis. Mutations would result in abnormal processing of such a factor, thereby disrupting normal P_i homeostasis. The endopeptidase may act on a variety of substrates, accounting for the multiple defects, or alternatively the P_i regulating factor may have variable functions at various target sites. Furthermore, exposure to chronic hypophosphatemia may have a role in the expression of other defects. Variability in bone disease may

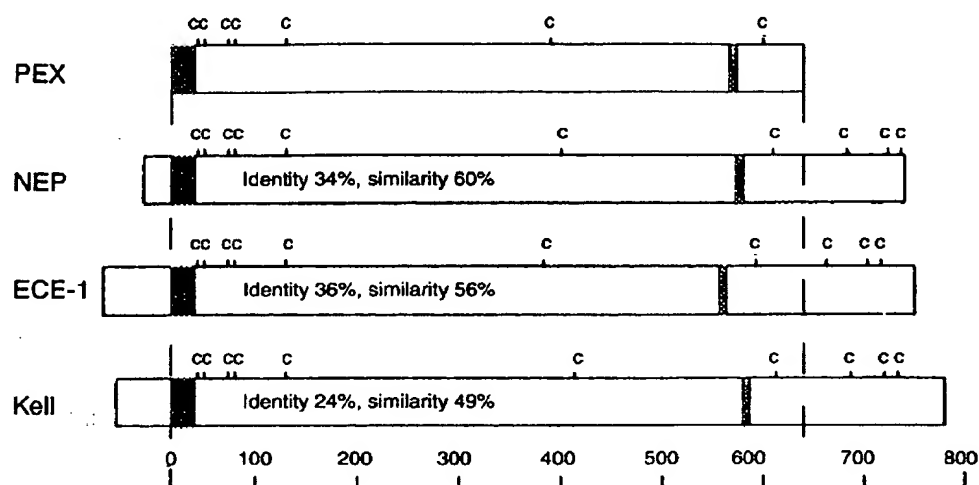


Figure 4. The predicted *PEX* gene product and homologous endopeptidases. Black regions represent the putative single membrane spanning domain. Gray regions depict zinc-binding motifs. c, Conserved cysteine residues. Amino-acid identity and similarity to the neutral endopeptidase (NEP), endothelin converting enzyme-1 (ECE-1), and the Kell blood group antigen (Kell) are noted. An amino-acid-numbering scale begins with position 1 of the predicted *PEX* sequence. (From HYP Consortium: A gene (*PEX*) with homologies to endopeptidases is mutated in patients with X-linked hypophosphatemic rickets. *Nat Genet* 11:133, 1995; with permission.)

be explained by variability in genetic background or the nature of a particular mutation in a given family.

TREATMENT

Although a varied experience is reported with medical intervention in patients with XLH, most investigators favor medical therapy. Despite important advances, treatment is not curative, and patients often manifest a variety of musculoskeletal symptoms, even with the best of attention. Goals of treatment include improvement in growth, and reduction in severity of bone disease, bow defects, and activity limitations. Therapy is not benign, and all physicians undertaking the long-term management of anyone with XLH should be aware of the potential complications of treatment.

Growth

The effect of medical treatment on height in XLH has been investigated by numerous investigators. On one side of the debate is the data from a retrospective study that suggested that treatment may be more harmful than beneficial and that no positive outcome with respect to height occurs.⁹³ Most investigators agree, however, that a positive effect on growth occurs with standard therapy with calcitriol [$1,25(\text{OH})_2\text{D}_3$] and phosphate salts. Earlier reports note an increase in height standard deviation (SD) score with use of calcitriol or $1\alpha\text{-OHD}_3$.^{19, 73} A

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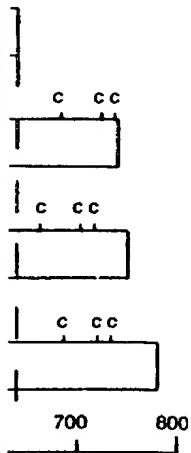
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randomized, long-term prospective trial of the effects of a standardized regimen on final adult height has not been performed, but lessons from available studies are pertinent. Verge and colleagues¹⁰⁴ demonstrated an efficacious response to therapy when compared with historical controls. In this study, girls tended to be better growers than boys. Balsan and Tieder⁷ have confirmed the importance of 1α -hydroxylated vitamin D metabolites with respect to height outcomes. A retrospective study by Seikaly and colleagues⁹⁹ suggested that vitamin D metabolites alone (without supplemental phosphate) may enhance growth. Other studies point out the large degree of variability in response to therapy. Petersen and colleagues⁶⁷ described good and poor growers in their study, and in agreement with Verge and colleagues,¹⁰⁴ a larger percentage of girls were in the good responder group. Friedman and colleagues³⁷ described variable responses to treatment in the North Carolina population, with outcomes highly dependent on height percentile at onset of therapy. Such findings suggest that early years of this disease are important in the genesis of the stature deformity. Others have argued that the degree of final height impairment is, in part, related to the severity of disease throughout childhood.⁴¹ Most descriptions of early intervention strongly suggest that medical therapy in the first 2 years of life results in improved outcomes.^{60, 86, 87}

Bone Disease

As with growth effects, bone lesions seem to best respond to phosphate and 1α -hydroxylated vitamin D metabolites.^{38, 40, 73} Interestingly, a characteristic halo lesion around osteocytes does not improve with therapy, which may reflect an intrinsic defect of this cell type.²¹ The author's experience is in agreement with the widely held, but undocumented opinion, that fewer osteotomies have been performed for severe bone disease since the availability of calcitriol.

Recommendations for Medical Management of XLH

Age at Onset

The author finds that early treatment is likely to result in better outcomes. Therefore, early diagnosis is important. Candidate children in affected families should be screened for abnormal serum and urine phosphorus levels and serum alkaline phosphatase activity within the first month of life and at 3 and 6 months of life. Any result suggestive of XLH should be followed by radiographic examination. If these features are at all suggestive of rickets, the author begins therapy with calcitriol (Rocaltrol) and phosphate.

Initial Doses

Starting doses for an infant in the first 2 years of life are generally, for calcitriol, 0.25 μ g once or twice daily, with 250 to 375 mg of elemental phosphorus provided in 2 to 3 divided dosages. In early infancy, the contents of a calcitriol capsule can be extracted using a 1-mL syringe and an 18-gauge needle. The oil-based liquid can be administered to the child in formula or directly into the child's mouth from the syringe after removal of the needle. By 18 months of age, parents can usually supervise swallowing the entire capsule. Phosphate preparations used later in childhood are not convenient in infancy. We use

Phospha-Soda (Fleet's) solution, which contains 127 mg of elemental phosphorus per mL. An older child may use Neutra-Phos or Neutra-Phos K powder (250 mg elemental phosphorus per packet or capsule) dissolved into water, drinking the solution at intervals through the day. When the child is old enough to chew or swallow a tablet, the author prefers K-Phos Neutral, which contains 250 mg of elemental phosphorus per tablet.

Monitoring

Approximately 2 weeks after the onset of therapy, serum calcium and PTH levels, and urinary calcium and creatinine excretion should be assessed. It is important to maintain normal levels of serum calcium concentration and urinary calcium excretion, without engendering hyperparathyroidism. In small children, sampling should be repeated every 3 months. During stable growth periods, without dose changes or complications, frequency of visits may be reduced to every 4 to 6 months. After 1 year of therapy, and every 2 to 3 years thereafter, a renal sonogram should be performed to assess the development of nephrocalcinosis. After cessation of growth, or long-term stability of the appearance of the kidneys, frequency of ultrasonographic examination can be decreased. Throughout childhood, radiographs of the knees should be performed every 1 to 2 years to assess the need for medication adjustments.

Adjustments in Dosage

The dosage range in XLH patients at Yale-New Haven Hospital is wide, dependent on severity of the rickets, response to therapy, and complications encountered. An important principle of therapy is to maintain an appropriate balance in the use of the two types of medication. Marked overuse of calcitriol results in hypercalcemia and hypercalciuria, whereas unopposed phosphate increases may result in secondary hyperparathyroidism. Thus increases in dosage to improve healing, when no side effects are evident, should consist of a balanced increase in both calcitriol and phosphate. Minimal increments in dosages of each agent are prescribed and follow-up laboratory investigation should be performed within 1 month of the change in dose. It is advised to closely monitor patients beginning just before an anticipated pubertal growth spurt. The rapid growth occurring with puberty may result in considerable worsening of bowing defects, and a transient increase in medical therapy may be advantageous. The author resumes monitoring patients at 4-month intervals through puberty until cessation of growth.

In the author's hands, calcitriol is used in later childhood at dosages averaging 0.75 μg per day, and rarely exceeding 1.5 μg per day. In the author's population, 10 to 50 ng/kg/d of calcitriol is the usual bodyweight normalized dosage; however, because the responses to therapy are so variable, we do not place much value in the ascertainment of dose for a given individual by body weight. In fact, most patients are on the lower end of the range (10–25 ng/kg/d) except for transient increases during puberty. In the event of suboptimal healing of the epiphyseal lesions seen on radiographs of the legs, the dose of calcitriol is increased, usually in conjunction with an increase in phosphate. Elevated PTH values, without hypercalcemia or hypercalciuria, calls for an increase in calcitriol alone. In the event of hypercalciuria or hypercalcemia with normal PTH values, borderline vitamin D intoxication may be present, indicating the need for a decrease in calcitriol dose, or if severe, temporary cessation of all therapy. After the end of the growth spurt, and achievement of final height,

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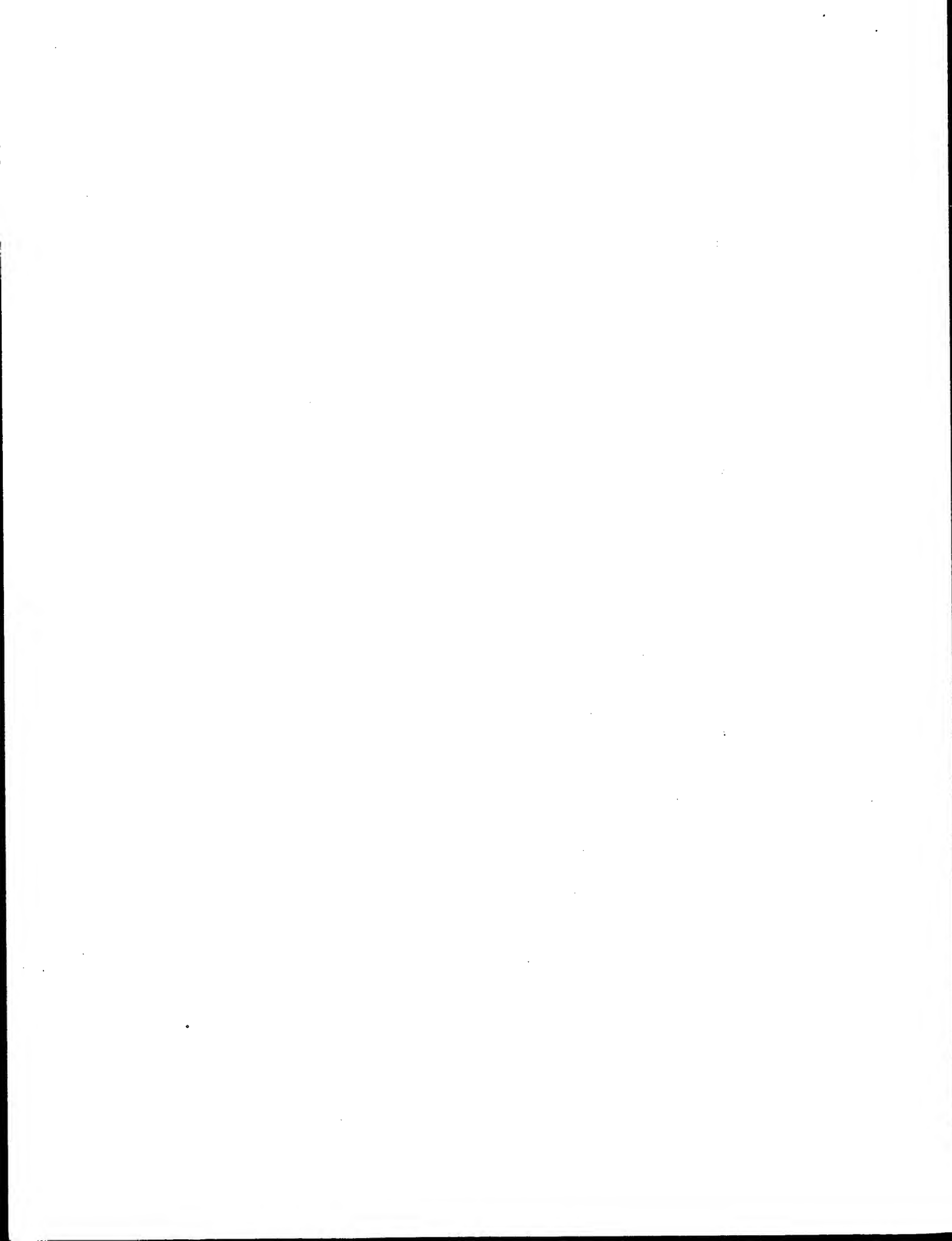
Dosages of elemental phosphorus average 1 g/d and rarely exceed 2 g/d. Diarrhea, bloody stools, or persistent dose-related abdominal pain indicate a need for reassessment of schedule of administration, and a probable reduction in total phosphate dose. The development of secondary hyperparathyroidism is an indication to alter the calcitriol/phosphate balance by decreasing phosphate or increasing the calcitriol dosage. In the event of a poor skeletal response, an increase in the dose of phosphate with a concomitant increase in calcitriol dosage is called for. Given the diurnal fluctuation in serum phosphorus levels, variability in kinetics of phosphate absorption, and the baseline hypophosphatemia in fasting patients with XLH, the author has not found it helpful to use serum phosphorus as an indicator of phosphorus demands. In fact, the presence of a decreasing serum phosphorus may incorrectly prompt an increase in phosphate dosage, when the underlying reason for the decreasing serum phosphate value is advancing hyperparathyroidism. The resultant increase in phosphate administration only worsens the hyperparathyroidism, and no improvement in serum phosphorus is likely. The author recommends administration of phosphate at three to four conveniently spaced intervals throughout the day. It is not necessary to awaken the child to provide medication through the night, as the diurnal rise in serum phosphorus occurs at night in patients with XLH, independent of therapy.¹⁶ Although some decrement in serum alkaline phosphatase activity usually occurs with therapy, this value rarely normalizes until adult age and is not a particularly good marker of disease even at that time. Therefore, although alkaline phosphatase activity may be of interest to periodically monitor, it is not always a sensitive indicator of therapeutic response.

Complications

Three major complications of therapy for XLH are: (1) hypervitaminosis D; (2) hyperparathyroidism; and (3) soft-tissue calcification, particularly in kidney. Significant clinical consequences of these complications can be avoided if patients undergoing therapy are carefully monitored. Indeed, treatment can be viewed as a compromise: to effectively achieve mineralization of the growth plate, the mineral load to the patient has to be increased. Yet this mineral load must not be so excessive as to result in significant soft-tissue calcification or derangement of parathyroid hormone function.

Hypervitaminosis D

Hypervitaminosis D is manifest by hypercalcemia and hypercalciuria. Its occurrence was more frequent with the use of high doses of fat-soluble vitamin D and infrequent monitoring of serum and urine biochemistries. 1 α -Hydroxylated vitamin D metabolites are more polar, are not stored in fat, and escape from toxic effects are more rapid than with native vitamin D. Although death from unrecognized vitamin D intoxication has occurred with this disease, it is much less common today than before the availability of 1 α -hydroxylated vitamin D metabolites.



Hyperparathyroidism

Hyperparathyroidism is not an uncommon occurrence in XLH; therefore, circulating PTH must be routinely monitored in individuals with the disease, particularly if receiving therapy. The stimulatory effect of P treatment on PTH secretion has been well-described in XLH^{8, 109}; phosphate supplementation should never be given as single therapy in the condition. Concomitant use of calcitriol dampens this PTH stimulatory effect of phosphate and should be part of the treatment regimen. Elevations in circulating PTH are more likely to be detected at night.¹⁶

Soft-tissue Calcification

Soft-tissue calcification, particularly at the level of the renal medullary pyramids (nephrocalcinosis), can be detected by ultrasonograms of the kidney. It has been suggested that this phenomenon is closely associated with the amount of phosphate salt the patient ingests.¹⁰⁸ Patients with XLH cared for at this institution demonstrate the finding within 3 to 4 years of beginning therapy. Poorly compliant patients have been less likely to develop the abnormality, suggesting a relationship to therapeutic intervention, as reported by Taylor and colleagues.⁹⁵ Most patients at this institution reach a modest grade of calcification, with limited progression to more severe stages. Occasionally, a slight decrease in severity occurs with time. This experience is similar to that of Kooh and colleagues,⁴⁶ who have reported 80% incidence of nephrocalcinosis in their patients with XLH and have seen no associated sequelae in patients with nephrocalcinosis present for up to 15 years. A decrease in glomerular filtration rate, however, was reported in one patient with hypertension and nephrocalcinosis.¹⁰⁵ The pathogenesis of the lesion is not clearly understood; some have suggested that increased urinary oxalate may occur with phosphate loading and that calcium oxalate precipitation is enhanced.⁷⁷ Others have shown that the lesions consist of calcium phosphate and can be reproduced in the *Hyp* mouse by administration of phosphate.⁴ In addition, increased PTH levels may predispose the tissue to calcification. Other related problems reported in patients with XLH include calcification of the entheses, which is not thought to be treatment-dependent; ocular calcifications¹²; and myocardial and aortic valve calcifications.³⁹

Surgery

Despite the advantages of medical therapy, some patients require surgical correction of a severe bow defect. The approach to surgery for XLH is physician dependent, but several points have been made in the literature. General recommendations for osteotomy in childhood suggest that the bow be so severe that irreversible progression of the bow defect is unavoidable with continued growth, even with medical therapy.²⁵ Others recommend that children fewer than 6 years of age do not undergo this procedure because bow defects at this age or less are usually not severe, and aggressive medical therapy may correct the deformity over time.⁸⁴ It has been the author's experience that healing of the osteotomy in young children may be prolonged. Surgical procedures vary: fixation with plating and stapling have been performed, and more recently external fixation devices have been used.⁴³ One report emphasizes that, in adults, fixation by intramedullary nail or rod placement achieves optimal results.³⁵ An

orthopedic surgeon should be consulted for surgical intervention because of the need for optimal medical management of the osteotomy. Reoperation is necessary in patients with

Other Therapy

Diuretics

Thiazide diuretics are used in patients with XLH to increase calcium reabsorption. Patients with XLH who have not responded to medical therapy have not

Human Growth Hormone

Human growth hormone therapy has been used in improved growth in a long-term study.¹⁰⁸ In a study of predominantly male patients, the control group showed no improvement in growth, suggesting that the hormone therapy is effective.

Vitamin D Analogues

Nonhypophosphatemic vitamin D analogues are used in patients with XLH to improve bone development. In a study using 25(OH)D₃, improvement in bone density was observed, but the use of vitamin D analogues as a substitute for phosphate in XLH are being

SUMMARY: FUTURE DIRECTIONS

The recent advances in the explanation of the pathogenesis of XLH

- Human growth hormone therapy in patients with XLH improves growth.
- The discovery of the role of phosphate in bone metabolism does not change the need for phosphate replacement in XLH.
- Product inhibition of the phosphate reabsorption in the proximal tubule is a key feature of XLH.

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orthopedic surgeon with experience in procedures in children with XLH should be consulted before any intervention. Medical expertise through the course of surgical intervention is important. Medication dosages may require adjustment because of immobilization-associated hypercalcemia following osteotomy, but optimal medication schedules should be maintained to facilitate healing of the osteotomy. Recently, leg-lengthening procedures have been attempted in a few patients with XLH.¹⁰⁴

Other Therapeutic Measures

Diuretics

Thiazide diuretics or amiloride has been suggested as adjunctive therapy in patients with XLH.³ The goal of this measure is to increase renal calcium reabsorption and to enhance mineralization. The long-term effects of this measure have not been reported.

Human Growth Hormone

Human growth hormone in combination with standard therapy has resulted in improved circulating phosphate concentrations and height velocity in a short-term study¹⁰⁸ and a 3-year study in which a very short (Z-score = -3.4), predominantly male subject group was compared with a predominantly female control group with more typical short stature (Z-score = -2.1).⁸⁵ The latter study suggests that very short populations may benefit from adjunctive growth hormone therapy.

Vitamin D Analogues

Nonhypercalcemic vitamin D analogues have been considered as adjunctive therapy in patients with XLH to maximize skeletal effects and suppress the development of hyperparathyroidism. The recent study from the author's institution using 24,25(OH)₂D₃ in combination with standard therapy showed skeletal improvement and parathyroid suppressive effects.¹⁵ Studies examining this metabolite as a specific therapy for secondary hyperparathyroidism in patients with XLH are being performed.

SUMMARY: HYPOTHETICAL MODEL OF DISEASE

The recently established findings that should be recognized in a mechanistic explanation of pathophysiology in XLH/*Hyp* include the following:

- Human and murine studies suggest that a humoral factor mediates the defect in renal phosphate transport. Many tumors that produce such a factor are of primitive mesenchymal origin and are not infrequently found in bone.
- The disease of mineralized tissues (bones and teeth) seem to be independent of disease expression at the kidney (as reflected by circulating phosphate levels). Mineralized tissues may reflect a gene dose effect, which does not seem to be present in kidney.
- Production of 1,25(OH)₂D is not appropriate for the ambient hypophosphatemia.

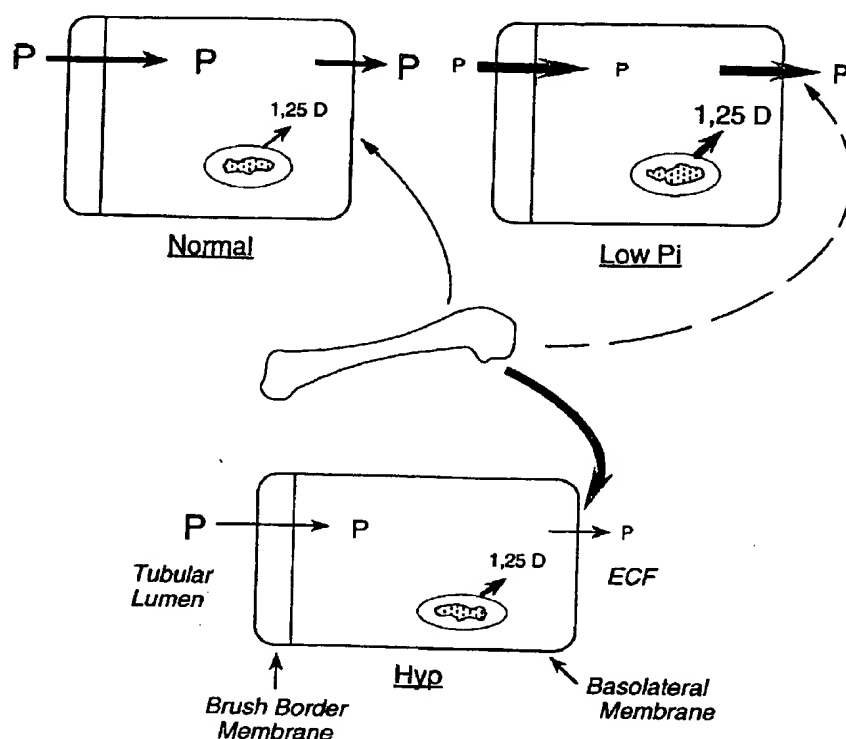


Figure 5. A hypothetical model of phosphate movement across renal cell membranes in Pi deprivation and *Hyp*. Pi deprivation initiates a signal resulting in basolateral efflux of Pi from the intracellular space into the serum; the resultant decrease in intracellular Pi serves as the signal for increasing brush-border transport of Pi, thereby decreasing urinary Pi losses. At the same time, the decrease in intracellular Pi serves as the signal for the stimulation of 1α -hydroxylase activity, and the resultant production of $1,25(\text{OH})_2\text{D}$ enhances intestinal Pi absorption and increases bone resorption, thereby increasing the serum Pi levels. Size of "P" represents relative Pi concentration in luminal fluid, intracellular space, and extracellular space; size of arrows through the cells indicate representative transport activity at each membrane; curvilinear arrows represent the postulated activity of the proposed factor affecting basolateral membrane Pi efflux.

- The mutated gene in XLH seems to encode an endopeptidase, and although expressed abundantly in murine bone relative to other tissues, it is underexpressed in *Hyp* mice.
- Renal intracellular P is probably normal, which may mask the hypophosphatemic stimulus to renal $1,25(\text{OH})_2\text{D}$ production.

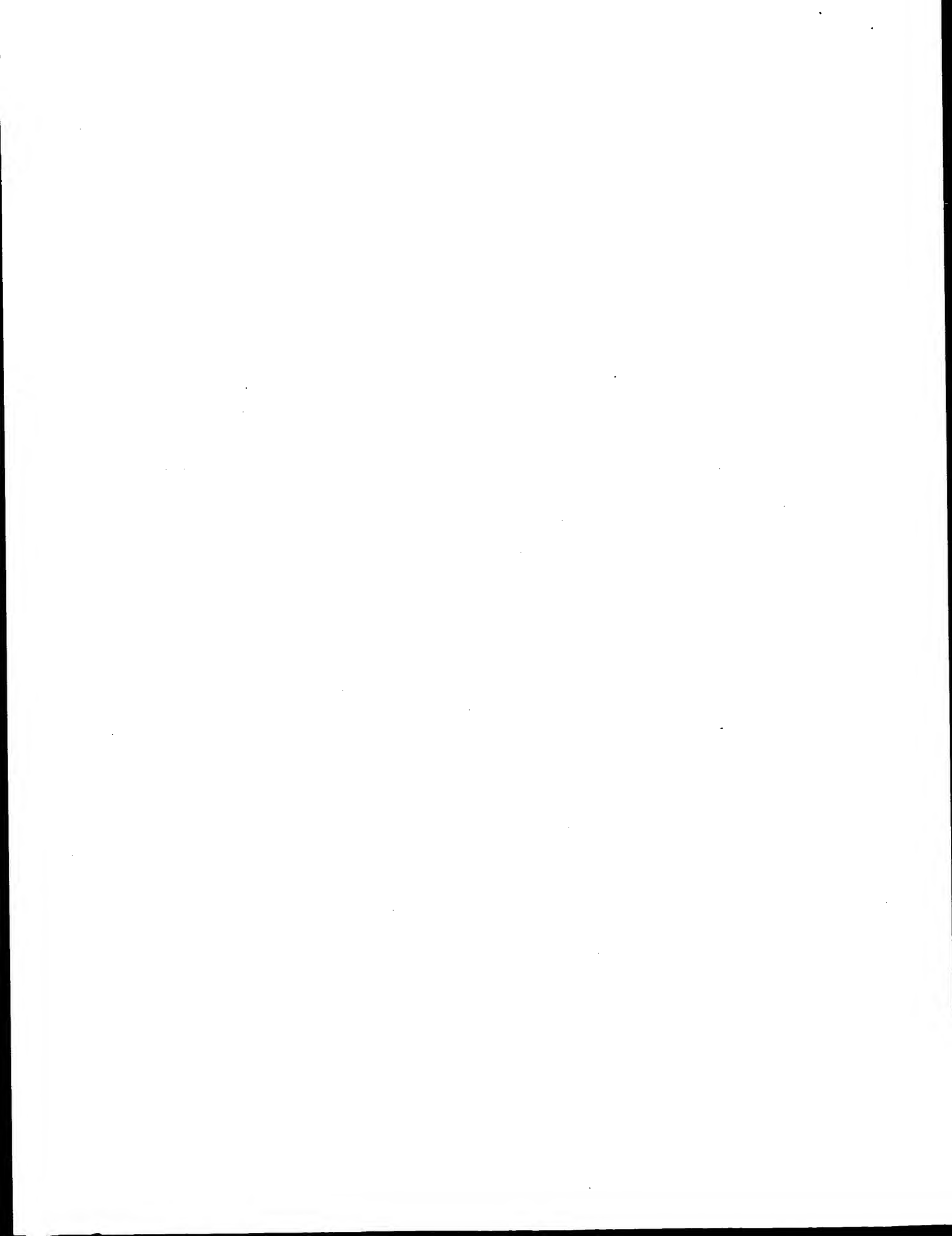
A unifying hypothesis that incorporates these findings is as follows: A putative Pi-regulating substance may be secreted by bone cells (as in OHO tumors). The author postulates that in normal steady state times of Pi sufficiency, elicitation of such a factor may preserve intracellular Pi at the proximal renal tubular cell. Luminal membrane Pi transport and 1α -hydroxylation of vitamin D would therefore be normally maintained. During Pi insufficiency, a decrease in the factor would decrease intracellular Pi, thereby mediating an increase in

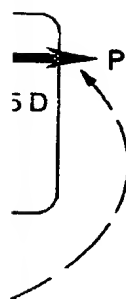
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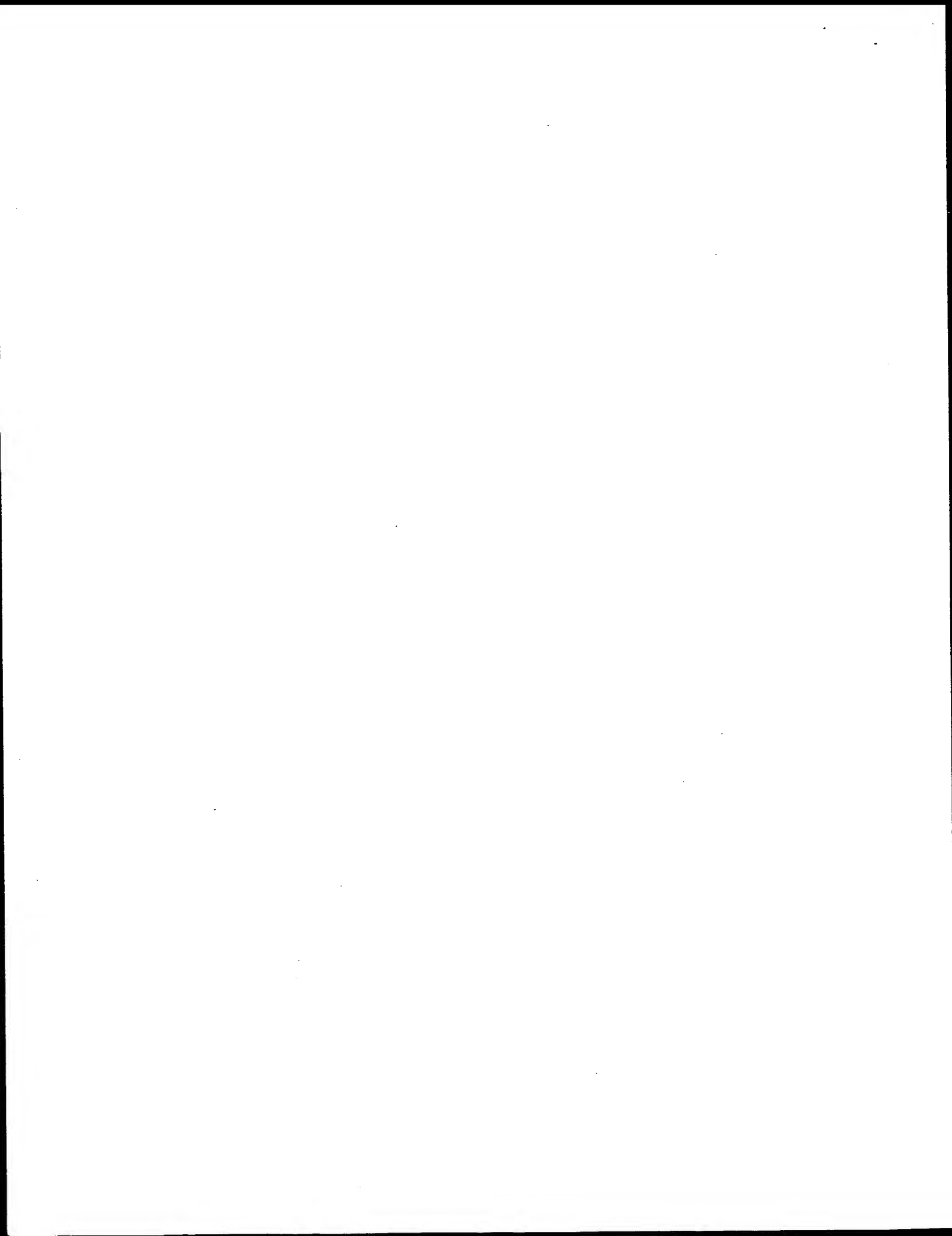


luminal membrane transport of Pi and 1 α -hydroxylation of vitamin D. Unregulated persistence of such a factor during brief Pi deprivation would disrupt appropriate renal Pi conservation and generation of 1,25(OH) $_2$ D, the precise renal defects seen in XLH/Hyp.

The author suggests that the synthesis, processing, or secretion of this putative humoral factor is rendered abnormal in XLH by mutations in the *PEX* gene, such that normal regulation of the Pi homeostatic system is disrupted. Further levels of complexity may exist: the renal-active factor may act directly on bone cells in an autocrine or paracrine fashion, or inappropriate regulation of intracellular Pi at the parathyroid cell may occur, resulting in the tendency to hypersecretion of PTH. Such a system would provide a mechanism by which skeletal mineral demands can be regulated at the renal level. Although this would be of physiologic service to the organism, no such system has been clearly established. This speculative hypothesis is summarized in Figure 5. Finally, others have suggested that a humoral factor interacts with receptors on the luminal membrane of renal tubular cells, increasing protein kinase C activity, which in turn results in the described renal tubular defects.⁷⁴

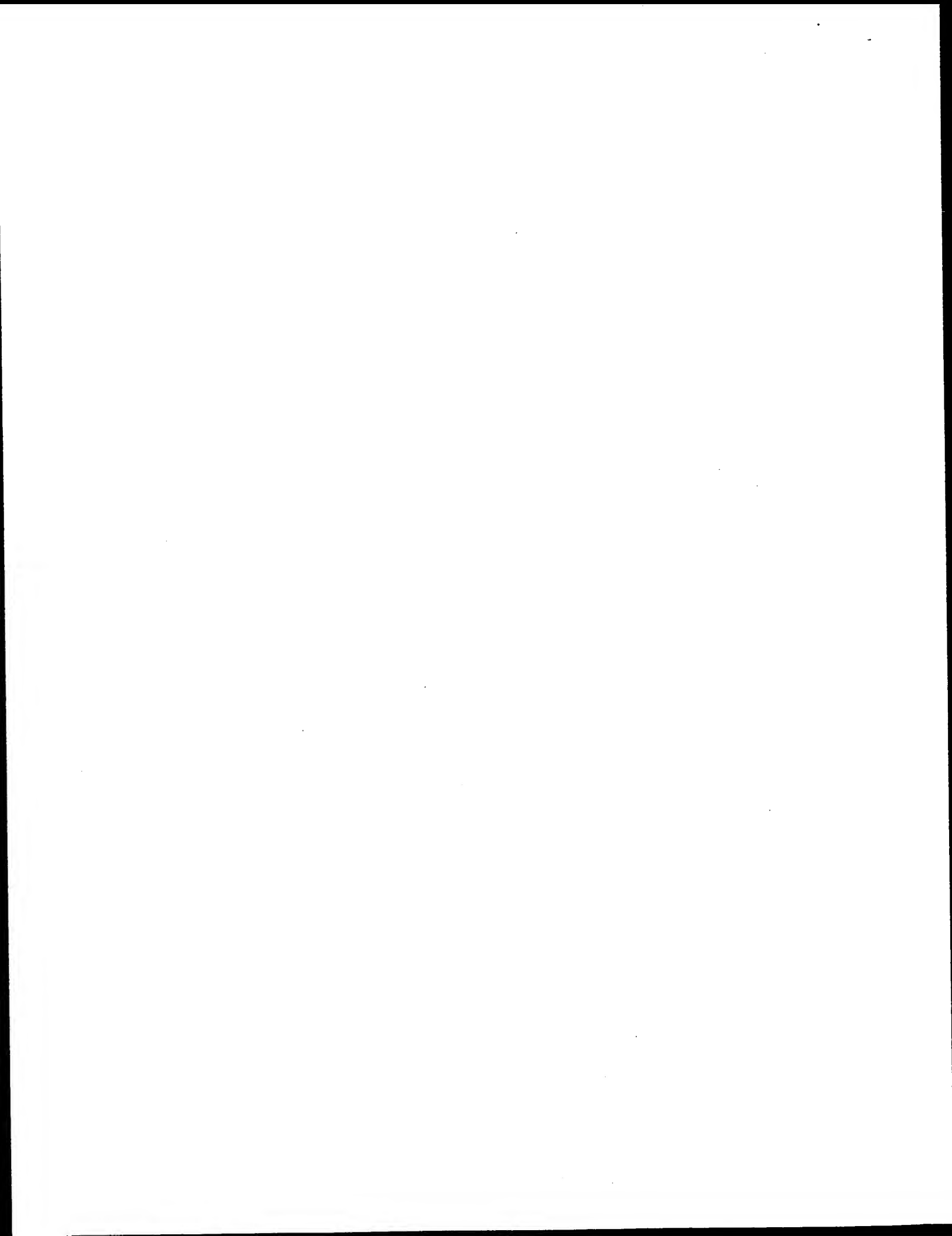
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Circadian rhythms of approximate 24 hours are observed in many organisms.^{65, 66} Notable examples include daily rhythms in the pathogenesis of myocardial infarction.

The system that generates these rhythms is the circadian clock, input pathways of which are located in the suprachiasmatic nuclei (SCN) in the hypothalamus.¹ The SCN express circadian rhythms approximately 24 hours in duration.

Lesion studies have identified the SCN as the site of a circadian clock. Transplantation of SCN cells into a host animal restores the day-night rhythmicity to the host.^{121, 126} Most rhythms are controlled by the SCN clock.⁷⁷ Most rhythms are controlled by the SCN cells.¹³⁷

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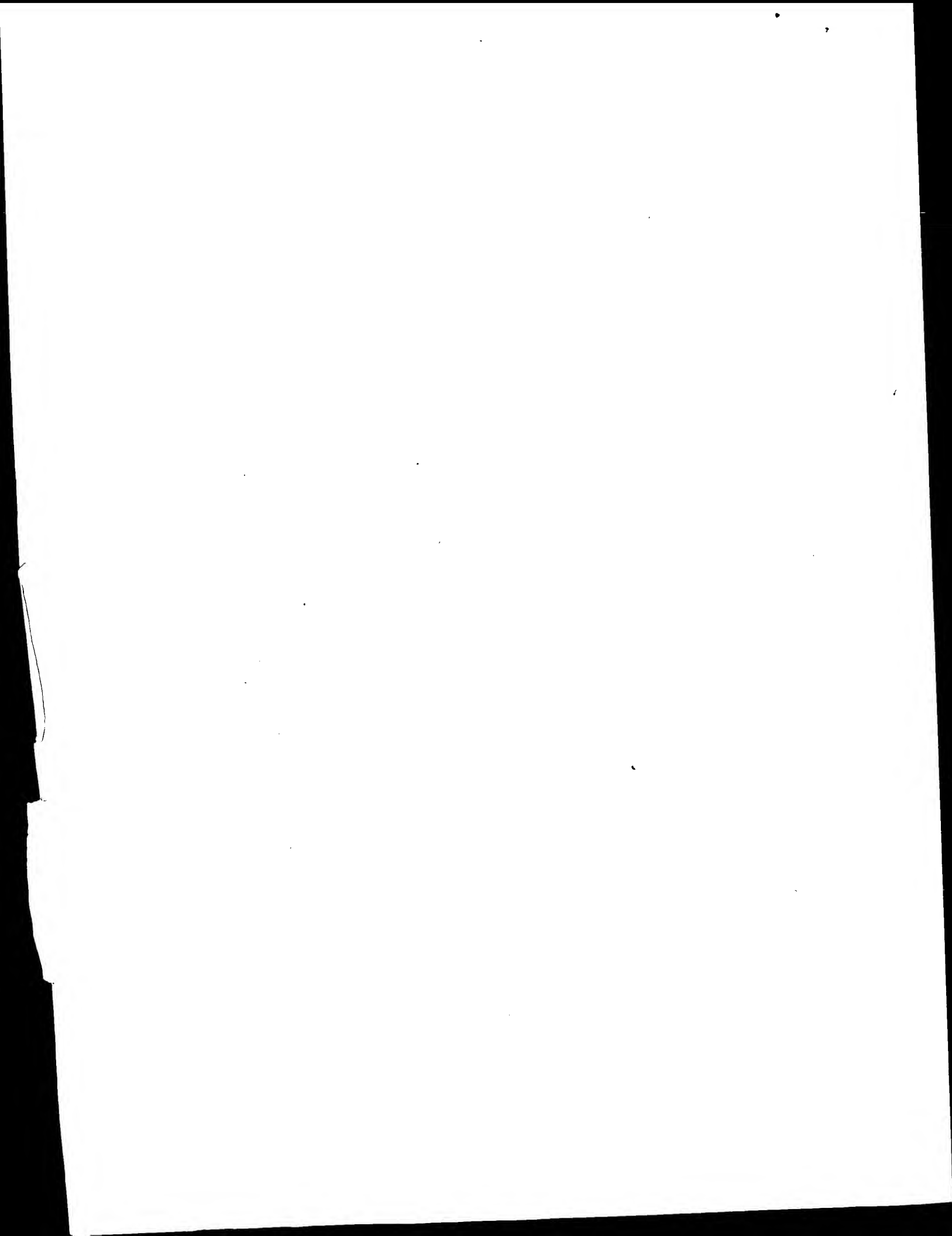
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Spermine deficiency in Gy mice caused by deletion of the spermine synthase gene

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Two mouse mutations *gyro* (*Gy*) and *hypophosphatemia* (*Hyp*) are mouse models for X-linked hypophosphatemic rickets and have been shown to be deleted for the 5' and 3' end of the mouse homolog of *PHEX* (phosphate regulating gene with homologies to endopeptidases on the chromosome; formerly called *PEX*), respectively. In addition to the metabolic disorder observed in *Hyp* mice, male *Gy* mice are sterile and show circling behavior and reduced viability. The human *SMS* (spermine synthase) gene maps ~39 kb upstream of *PHEX* and is transcribed in the same direction. To elucidate the complex phenotype of *Gy* mice, we characterized the genomic region upstream of *Phex*. By establishing the genomic structure of mouse *Sms*, a 160–190 kb deletion was shown in *Gy* mice, which includes both *Phex* and *Sms*. There are several pseudogenes of *SMS/Sms* in man and mouse. Northern analysis revealed three different *Sms* transcripts which are absent in *Gy* mice. Measurement of polyamine levels revealed a marked decrease in spermine in liver and pancreas of affected male *Gy* mice. Analysis of brain tissue revealed no gross or histological abnormalities. *Gy* provides a mouse model for a defect in the polyamine pathway, which is known to play a key role in cell proliferation.

INTRODUCTION

Gy (1) and *Hyp* (2) mice constitute mouse models for X-linked dominant hypophosphatemic rickets and are caused by mutations in the *Phex* gene (formerly called *Pex*) (3–5). In accordance with the phenotype observed in humans, both mouse mutants have renal phosphate wasting, impaired mineralization and growth retardation (1). Affected *Gy* males and some affected female

animals also show inner ear abnormalities, deafness, hyperactivity and circling behavior (1,6,7). Male *Gy* mice are also sterile (8), are smaller than their normal sibs from birth onwards and weigh less than *Hyp* males (1). The *Gy* mouse is bred on the B6C3H background and the *Hyp* mouse is bred on the C57BL/6J background. Interestingly, male *Gy* mice do not survive on the C57BL/6J background (9) and have a reduced viability from birth, with sudden death occurring in adults on the B6C3H background (1).

The *Gy* deletion extends upstream of the 5'-untranslated region (5'-UTR) of *Phex* (5). It seemed likely that the additional symptoms in *Gy* mice are caused by a contiguous gene deletion syndrome which includes at least one additional gene to *Phex*. We have mapped the human *SMS* gene distal to *PHEX* (formerly called *PEX*), and have shown that both genes have the same orientation (5). The genomic organization of the region containing *SMS* and *PHEX* has been reported (10) (GenBank accession nos: U53331, U72789, U72790, U73024 and Y10196). Human *SMS* is therefore known to be 39 kb upstream of *PHEX*.

Sms constitutes an attractive candidate gene for the additional symptoms in *Gy* mice because polyamines are required for normal growth and differentiation. The polyamines spermidine and putrescine are found ubiquitously in prokaryotic and eukaryotic cells whereas spermine is rarely found in prokaryotes but is widespread in eukaryotes [for review, see refs (11–14)]. Synthesis and degradation of polyamines are tightly regulated (15). Putrescine is synthesised from L-ornithine by ornithine decarboxylase, the rate-limiting enzyme in polyamine synthesis. Putrescine and decarboxylated S-adenosylmethionine are substrates for the synthesis of spermidine, which is converted to spermine by spermine synthase (16) (Fig. 1). Polyamines are organic polycations which interact with a variety of intracellular targets including nucleic acids, phospholipids and proteins. Putrescine and spermidine are known to play specific roles in the maturation of the eukaryotic translation initiation factor eIF-5A (17). Furthermore, specific interactions of spermine and

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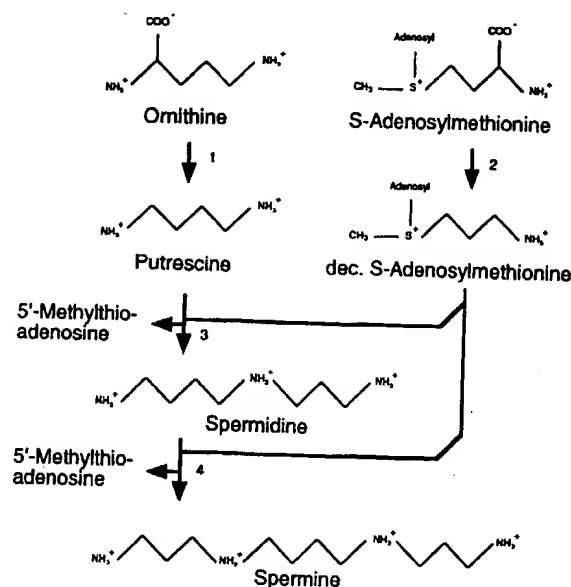


Figure 1. Polyamine biosynthesis pathway. Key to enzymes: 1, ornithine decarboxylase; 2, S-adenosylmethionine decarboxylase; 3, spermidine synthase; 4, spermine synthase.

spermidine with certain potassium channels and glutamate receptors have been reported (18–20).

The homologous mouse sequence of *SMS* has been isolated from an embryonic cDNA library, and preliminary analysis gave no evidence that sequences at the 3' end are deleted in *Gy* mice (5). We have now determined the genomic organization of the mouse *Sms* gene and show that *SMS/Sms* has several pseudogenes. The *Sms* gene is deleted in *Gy* mice, providing the first mouse model for a defect in the polyamine pathway.

RESULTS

SMS/Sms pseudogenes

Screening a mouse P1 library with *Sms* cDNA revealed five different clones (703K15175, 703K23169, 703D07320, 703E04130 and 703C14122). PCR with primers from exon 2 (SpSyF3) and the 3'-UTR (SpSyR1) using P1 DNA as template revealed discrete products of ~1400 bp, suggesting the presence of pseudogenes which have arisen by integration of reverse-transcribed RNA. The PCR products were sequenced and all of them showed deletions and insertions causing frameshifts. Southern blot analysis of *Eco*RI digests of genomic mouse DNA showed various bands between 1 and 14 kb. The absence of dosage differences between male and female genomic DNA on Southern blots suggests the existence of several autosomal pseudogenes in humans (data not shown).

Sms is deleted in *Gy* mice

In order to characterize the distal deletion breakpoint in *Gy* mice, we then isolated a mouse bacterial artificial chromosome (BAC) clone, 308A23, by PCR screening using primers from the 5'-UTR of *Phex*. Restriction enzyme digestion of 308A23 gave a size of

~150 kb. Hybridization of various *Phex* and *Sms* RT-PCR products to the BAC DNA showed the presence of the entire *Sms* gene and of exon 1 of the *Phex* gene. Intron-exon boundaries of *Sms* exons were determined by preparing a shotgun library of 308A23 and sequencing clones which hybridized with the mouse *Sms* cDNA (5). The BAC ends were determined by direct sequencing and by sequencing of products generated by vector-Alu PCR. In addition, a 1.5 kb *Hind*III SP6 end fragment was subcloned and sequenced. The T7 end lies on the same 4.3 kb *Bam*HI end fragment as the exon 1 of *Phex*. Partial restriction mapping indicated that the BAC clone extends from intron 1 of the *Phex* gene to ~56 kb upstream of the first exon of the *Sms* gene (Fig. 2).

SMS/Sms is highly conserved between man and mouse. The open reading frame (ORF) comprises 1101 nucleotides. The mouse gene shows 91% identity at the DNA level and 96% identity on the protein level to the human gene. The mouse gene comprises 11 exons showing the same exon-intron structure as the human homolog (GenBank accession no. U53331).

PCR amplification from *Gy* genomic DNA with intronic primers of the 5'-UTR, exons 2, 4, 7 and 10 revealed a deletion of the entire *Sms* gene. The T7 end of the BAC clone was shown to be deleted while the 1.5 kb *Hind*III SP6 end fragment was present, which was confirmed by sequencing the PCR products and hybridization to *Hind*III digest of genomic *Gy* DNA. Hybridization of the 1.5 kb *Hind*III fragment to genomic mouse *Eco*RI Southern blots revealed an ~6.5 kb junction fragment in *Gy* mice, instead of an 11 kb genomic *Eco*RI fragment in control mice (Fig. 3). Furthermore, a 2.5 kb *Eco*RI fragment, which lies ~34 kb distal to exon 1 of the *Sms* gene, was subcloned and sequenced. PCR amplification with primers from this sequence showed that the deletion in *Gy* extends up to this *Eco*RI fragment. Thus, the distal deletion breakpoint of the *Gy* deletion lies ~50 kb distal to exon 1 of the *Sms* gene.

Sms expression

Sequencing of cDNA clones isolated from a mouse embryonic cDNA library revealed two variant transcripts, with exons 3 and 4 absent or present. Poly(A)⁺ RNA from normal mouse embryos and from adult *Gy* mice and controls was subjected to northern analysis using *Sms* cDNAs as probes. A northern blot (Clontech) containing RNA from total mouse embryos of day 7, 11, 15 and 17 was hybridized with a full-length mouse cDNA probe and separately with a probe of exons 3 and 4. In both cases, three transcripts of 2, 3 and 4 kb were detected (Fig. 4A). The 3 kb transcript produced only a weak signal. The highest expression could be detected on embryonic day 11.

RNA was extracted from male B6C3H *Gy* tissue. RNA from male unaffected littermates was used as control. Spleen expressed the 2 and 3 kb transcripts, brain expressed the 2 and 4 kb transcripts. Even exposure of the blot to autoradiograph film for 2 weeks gave no evidence of a 4 kb transcript in spleen tissue and a 3 kb transcript in brain tissue. In *Gy* mice, no *Sms* expression could be detected in brain or spleen tissues (Fig. 4B). The same data were obtained using probes comprising exons 2–11 as well as exons 9–11. Thus, none of the transcripts detected on northern blots are caused by processed pseudogenes or *Sms* homologs because all three are absent in *Gy* mice.

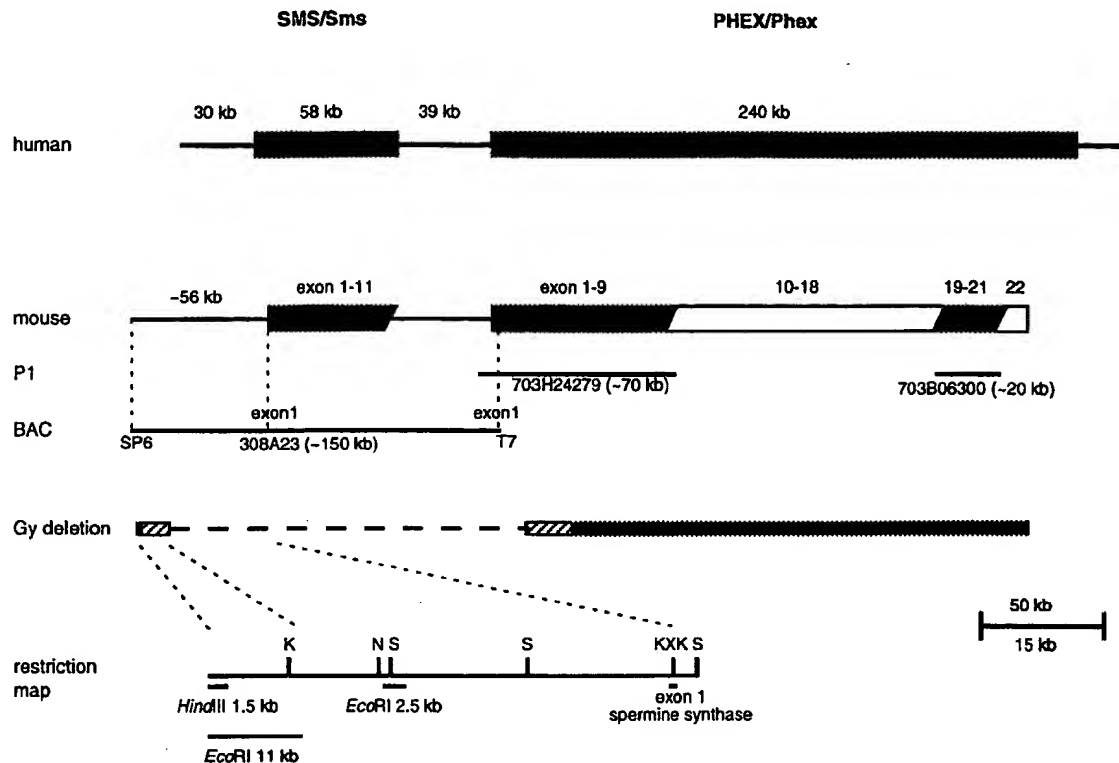


Figure 2. Genomic organization of the *SMS/Sms* and of *PHEX/Phex* genes. In the top line, the genomic organization of the human genes is drawn to scale. The genomic distances are derived from the sequence of this region. In the second line, the genomic organization of the syntenic region in mice is shown. The regions of the mouse genes, which are covered by genomic clones, are indicated by black boxes. Exon 1 of the *Phex* gene lies on the 4.3 kb *Bam*HI end fragment of BAC 308A23. Exon 1 of the *Sms* gene lies ~56 kb downstream of the SP6 end of BAC 308A23. Below, the deletion in *Gy* mice is shown, comprising 160–190 kb. The dotted line indicates the deleted region, black boxes indicate the region present in *Gy* and the hatched boxes indicate the regions which bear the deletion breakpoints. Hybridization of the 1.5 kb *Hind*III SP6 end fragment to genomic mouse *Eco*RI Southern blots revealed an ~6.5 kb junction fragment in *Gy* mice, instead of an 11 kb genomic *Eco*RI fragment in control mice. The proximal deletion breakpoint lies within intron 3 of the *Phex* gene. The BAC clone 308A23 was partially restriction mapped. The bottom line shows the restriction map from the SP6 end up to exon 1 of the *Sms* gene (X, *Xho*I; N, *Nru*I; K, *Ksp*I; S, *Sal*I).

Table 1. Tissue concentrations of polyamines in 12-day-old male *Gy* mice (1,2), control littermates (3–5), male *Hyp* mice (6,7) and control littermates (8,9)

No.	Mouse	Putrescine		Spermidine		Spermine	
		Liver	Pancreas	Liver	Pancreas	Liver	Pancreas
1	<i>Gy/Y</i>	0.063	0.253	1.77	4.26	n.d.	0.030
2	<i>Gy/Y</i>	0.194	0.083	2.55	4.22	n.d.	0.015
3	+/ <i>Y</i>	0.111	0.193	1.87	3.42	0.165	0.342
4	+/ <i>Y</i>	0.098	0.237	2.53	3.42	0.378	0.329
5	+/ <i>Y</i>	0.094	0.273	1.44	3.62	0.204	0.392
6	<i>Hyp/Y</i>	0.054	0.098	1.54	4.14	0.602	0.364
7	<i>Hyp/Y</i>	0.141	0.272	1.81	2.98	0.379	0.314
8	+/ <i>Y</i>	0.039	0.122	1.76	5.81	0.549	0.439
9	+/ <i>Y</i>	0.104	0.299	1.72	3.20	0.355	0.370

n.d. = not detectable.

The concentrations of putrescine, spermidine and spermine are given in $\mu\text{mol/g}$ fresh weight.

Polyamine levels

Polyamine levels were measured by reversed phase HPLC after pre-column derivatization with benzoyl chloride, in liver and pancreas of 12-day-old male affected *Gy* mice ($n = 2$), male control littermates ($n = 3$), male affected *Hyp* mice ($n = 2$) and control littermates ($n = 2$). In male affected *Gy* mice, the spermine levels were clearly decreased in pancreas and below the detection limit in liver. The putrescine and spermidine levels were not different between *Gy*, control and *Hyp* mice. Male affected *Hyp* mice showed normal spermine levels when compared with control male littermates (Table 1).

Histology

On day 12, autopsy was performed in two affected male *Gy* mice. At this time, the body weight of male *Gy* mice (4.5 and 4.8 g) was less than in control littermates (6.0, 6.0 and 7.3 g). Examination of brain tissues in male *Gy* mice showed no gross or histological abnormalities. Analysis of 12 serial sections of the mes- and metencephalon revealed a normal architectural organization of the cerebellar cortex and cochlear nucleus and gave no evidence for a decreased number of granule cells, which have been reported in rats and hamsters treated postnatally with the inhibitor of

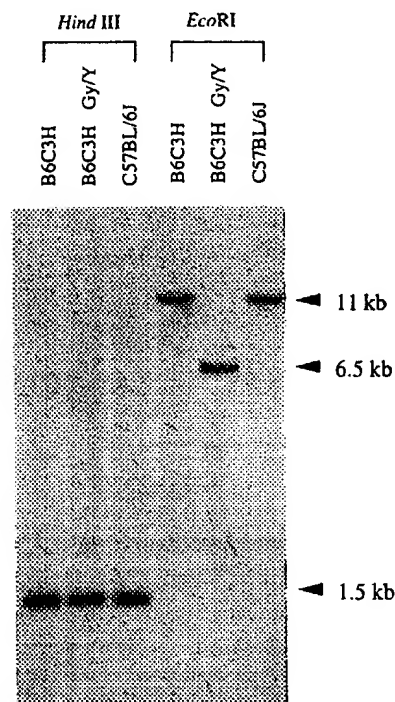


Figure 3. Southern blot of *Hind*III and *Eco*RI digests of mouse genomic DNA. Hybridization with the 1.5 kb *Hind*III SP6 end fragment of BAC 308A23 revealed an ~6.5 kb junction fragment in *Gy* mice, instead of an 11 kb genomic *Eco*RI fragment in control mice.

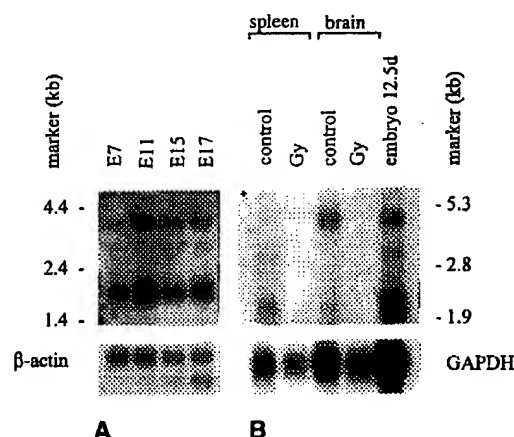


Figure 4. (A) A mouse embryo northern blot (Clontech) was hybridized with a full-length *Sms* cDNA probe (exposure: 4 days) and rehybridized with β -actin cDNA as control (exposure 1 day). (B) *Sms* RNA expression in male B6C3H *Gy* mice and unaffected male littermates. 2 μ g of poly(A)⁺ RNA from adult spleen, brain and 12.5 day total embryo was electrophoresed in a formaldehyde-agarose gel, transferred to a nylon membrane and hybridized with an *Sms* cDNA probe comprising exons 2–11 (exposure: 2 days). The same filter was rehybridized with a GAPDH cDNA probe to control for RNA loading (exposure: 12 h).

L-ornithine decarboxylase, DL- α -difluoromethylornithine (DFMO) (Fig. 5). At autopsy, aside from rickets, no other gross or histological abnormalities were observed. In particular, a histological examin-

ation of the heart, thymus, kidney, liver and spleen revealed no obvious abnormalities. Testes have not yet been examined in this study.

DISCUSSION

Gy and *Hyp* are mouse models for X-linked dominant hypophosphatemic rickets bearing deletions of the *PheX* gene. The disease shows a similar phenotype in man and mouse. In both species, low serum phosphate levels are caused by a defect of the proximal tubular phosphate reabsorption, causing rickets and osteomalacia. In spite of low phosphate levels, serum vitamin D levels are not elevated but are in the normal range. The phenotype in females is as severe as in males. Male *Gy* mice, however, show additional symptoms (1): they have a reduced viability whereas affected men and male *Hyp* mice have a normal viability. Male *Gy* mice are smaller from birth onwards, whereas in man and in *Hyp* mice birth weight is normal and short stature is acquired during postnatal life. Male *Gy* mice are sterile whereas male *Hyp* mice are fertile and have only been reported to possibly have a reduced fertility (1). Apart from these clearly distinguishable symptoms, there may be some overlap concerning the circling behavior. *Gy* males and some affected females show circling behavior, and this has also been observed occasionally in *Hyp* mice on the B6C3H background (9). *Gy* mice are bred on the B6C3H, *Hyp* mice on the C57BL/6J background. Nevertheless, the differences in viability, birth weight and sterility of *Hyp* and *Gy* mice do not seem to be related to modifier gene effects: when *Gy* mice are transferred to the C57BL/6J background, affected males do not survive (9). The *Gy* phenotype suggests that, apart from hypophosphatemia, an X-linked recessive trait is inherited in this mouse model.

We show that, in addition to *PheX*, the *Sms* gene is deleted in *Gy* mice. The BAC clone 308A23 covers ~150 kb and contains the entire *Sms* gene. It extends from intron 1 of the *PheX* gene to ~56 kb upstream of the first exon of the *Sms* gene. We have determined a partial genomic sequence of the mouse *Sms* gene, showing that the genomic organization of the mouse gene is comparable with the human gene. Comparison with the human genomic sequence gave no evidence of further genes in between *PHEX* and *SMS* genes (U53331, U72789, U72790, U73024 and Y10196) and 30 kb upstream of the first exon of *SMS* (U73023 and U72787) (10). Thus, the additional symptoms in *Gy* seem to be caused by the deletion of the *Sms* gene.

Northern blot analysis of *Sms* shows three transcripts of ~2, 3 and 4 kb. All three transcripts are absent in *Gy* mice, which excludes the possibility that they are due to transcribed pseudogenes or homologs of *Sms*. During embryogenesis, *Sms* expression seems to be highest at day 11, suggesting a role for this enzyme in development. In adult mice, tissue-dependent expression was observed: the 4 and 3 kb transcripts are not expressed in spleen and brain, respectively. So far, we do not know whether the different transcripts are caused by alternative splicing or the use of alternative polyadenylation signals. A large number of *Sms* expressed sequence tags (ESTs) found in dbEST all seem to suggest the use of an unusual polyadenylation signal, ATTAAA, situated 410 bp downstream of the stop codon. This polyadenylation signal is conserved in human and mouse gene sequences, and predicts a 1.6 kb transcript size. However, a 2 kb transcript has also been reported in human northern blot experiments (16), and the differences between predicted and observed sizes are still

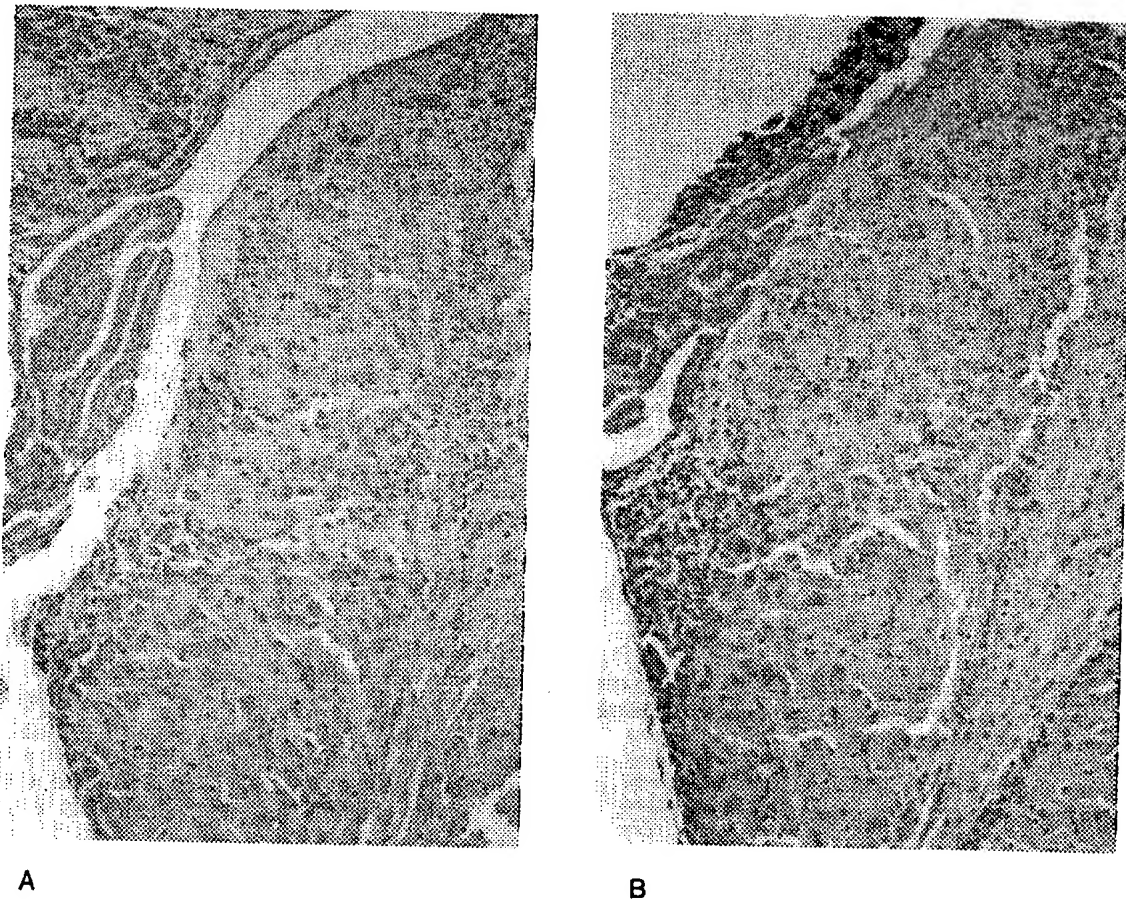


Figure 5. Photomicrographs of coronal sections of the hindbrain in 12-day-old mice: male Gy mouse (A), male control littermate (B). The top of the panel shows the dorsal cochlear nucleus and the bottom shows the ventral cochlear nucleus (magnification 160 \times , staining: HE). Both sections show comparable sizes of these nuclei and normal numbers of granule cells, giant cells and fusiform cells.

unclear. In humans, a second polyadenylation signal is predicted by GRAIL (23) to lie 809 bp downstream of the stop codon, but only a single truncated EST is present in dbEST which fits this site. It is known that spermine tissue levels differ markedly in different cells: they are high in prostate and seminal plasma, and low in brain and muscle (24). The presence of multiple transcripts and tissue-specific transcription patterns may contribute to the tissue-specific regulation of spermine levels.

Polyamine metabolism has been studied using inhibitors both *in vivo* and *in vitro*. Treatment with the highly selective, irreversible inhibitor of L-ornithine decarboxylase, DFMO, has revealed that the rate-limiting enzyme of polyamine synthesis has an essential role in mammalian embryogenesis. DFMO treatment arrests early stages of mammalian embryogenesis (25). When the dose or duration of treatment with DFMO is reduced, organ-specific growth deficits become apparent, affecting especially developing brain tissue (for review, see ref. 26). DFMO treatment causes near total depletion of putrescine and a marked decline in spermidine levels, but leaves spermine levels unaltered. This is a different pattern from that observed in Gy mice. There, putrescine and spermidine levels are not altered at day 12; only spermine levels are markedly decreased in pancreas and not detectable in liver. Since all mammalian cells are able to import polyamines (27), spermine levels in Gy mice may be less altered during

embryogenesis. During postnatal life, further changes in polyamine levels may occur.

Despite the differences in polyamine levels, there are some similarities in the clinical symptoms between Gy mice and DFMO-treated animals. Hearing impairment (6,7) has been reported in Gy mice and this was also found in DFMO-treated guinea pigs (28). Male Gy mice are small at birth, have a reduced viability on the B6C3H background and do not survive on the C57BL/6J background. No gross or histological abnormalities of brain tissue were detected at day 12. Particularly, no alteration of the morphology of the cerebellum and the dorsal cochlear nucleus was detected, which has been reported in DFMO-treated rats (26) and hamsters (29), respectively. Spermine deficiency may cause more subtle alterations such as changes in channel activity of certain potassium and glutamate receptors in heart or brain (18–20) or changes of the regulation of mitochondrial Ca^{2+} transport (30).

Recently, spermine synthase inhibitors have become available (31,32). So far, they have only been tested *in vitro*. Cell culture experiments have shown that the spermine content can be reduced profoundly and cell growth can be decreased in human breast cancer cells (33) and mouse leukemia cells (34). However, there have been no obvious effects on growth and viability in rat hepatoma cells (32). The Gy deletion provides a mouse model for

spermine synthase deficiency without the disadvantages of inhibitors such as unspecificity and toxicity. This model will allow the investigation of spermine deficiency and may help to unravel some of the mysteries of spermine function.

MATERIALS AND METHODS

Genomic clones

P1s were isolated by screening gridded mouse P1 library filters. This P1 library was prepared using *Mbo*I partially digested DNA obtained from C57BL/6 female mice spleen (F. Francis, unpublished). The cloning vector was pAd10sacBII and the host strain NS3145 (21). Primers SpSyF3 (GTG AGG CCA TTC TGA AAG GC) and SpSyR1 (CTA AGT CAA TTT GGG GGT GAG) were used to amplify the pseudogenes from P1 clones.

The BAC clone, 308A23, was isolated by PCR screening BAC DNA pools with primers from the 5'-UTR of *Phex* (PIMF1: GCT TGA GCA AAA AGC CTG CC; PIMR1: ACC AGG GTG CCA CCA ATA AAC) (Research Genetics Inc.).

cDNA libraries

A human cDNA clone containing the 3'-UTR of *SMS* was used to identify clones in mouse 9 day and 12 day embryonic libraries (B. Herrmann, unpublished).

Exon-intron structure of mouse *Sms*

A shotgun library of BAC 308A23 was constructed by ligation of sonicated BAC DNA to blunt-ended linearized plasmid DNA (pUC18). A total of 800 clones were gridded on filters and hybridized with the mouse *Sms* cDNA. Inserts of positive clones were amplified by PCR and sequenced from both sides using ABI dye primer chemistry. The following primers were used to amplify the 5'-UTR and exons 2, 4, 7 and 10: 5'-UTR, SSM5UTRF (GGA GAG GCA CAG CAA TCC ATA C) and SSM5UTRR (GCC TCG CTA ATG GTG GAA TCG); exon 2, SSM2F (TCT TTT TCC TAA TCG CCT TCC C) and SSM2R (CCA TGA GAT CAA CTT ACC TGC C); exon 4, SSM4F (GAC TAT GAT TTG GGC AGC AC) and SSM4R (CAT ATC TTC AGC AGA CAT TAA G); exon 7, SSM7F (CTT TAT GAT CCT CAG ATG GC) and SSM7R (TTA AAA GTA TGC TAC CAA GGG); exon 10, SSM10F (GAA TCC GCT AAA TTG CAT GGT G) and SSM10R (AGA TGC TCT GAA AAC GTA GCA C).

BAC ends

BAC ends were generated by direct sequencing of the BAC DNA and by sequencing of PCR products amplified with vector (T7/SP6) and Alu primers (CL1, TCC CAA AGT GCT GGG ATT ACA G; CL2, CTG CAC TCC AGC CTG GG). The following primers were used to amplify the BAC ends from genomic DNA: BACSP6F2 (CTG AAA AGT GCC ATT AAG GTG) and BACSP6R2 (GAA AGG GTC TTG CTA TGT GG); 1.5 *Hind*III SP6 end fragment, SP6F3 (GCT AGA CCT TGG TTG AAT GGA) and BACH15R (ACC TGA AAA GGT CTG TGC CTT); BACT7F (CCC TTT CTT TAC TGT CTG CCC) and BACT7R (GGT TTT CAG GTC CCA CCT CAG).

Sequencing

PCR-amplified products and genomic fragments cloned into plasmid vectors were purified by gel extraction and plasmid preparation kits (Qiagen). Cycle sequencing was performed using a *Taq* DyeDeoxy Terminator Cycle sequencing Kit (ABI). The sequences were determined with an Applied Biosystems 377 automated sequencer.

Northern blots

The mouse embryo northern blot was obtained from Clontech and hybridized according to the manufacturer's instructions. Total RNA from *Gy* mice was prepared using TRIzol reagent (GIBCO/BRL). Poly(A)⁺ RNA was then isolated from total RNA using an Oligotex mRNA mini kit (Qiagen). About 2 µg of poly(A)⁺ RNA from each sample was electrophoresed on a 1% formaldehyde-agarose gel and transferred to Hybond-N nylon membranes (Amersham). The filters were then hybridized with ³²P-labeled probes.

Polyamine measurement

Polyamines were determined by reversed phase HPLC after pre-column derivatization with benzoyl chloride (22). Dissected organs were snap-frozen in liquid nitrogen and pulverized in a microdismembrator. Diaminohexane (40 nmol) was added as internal standard and protein was precipitated with 0.2 M perchloric acid. After neutralization with 4 M KOH, polyamines were derivatized with benzoyl chloride, extracted with diethyl ether and dissolved in methanol:water (60:40 v/v). HPLC separation was performed on a Waters µBondapak C18 column (3.9×300 mm) with methanol:water (60:40 v/v) at a flow rate of 1 ml/min. The UV signal was monitored at 254 nm. Determination of polyamines displayed linearity over the whole range of tissue polyamine concentrations and was documented up to 5 nmol per 20 µl injection. Recovery of the internal standard ranged between 52 and 99%. Recovery of external standards after measurement of derivatized samples was 100–107%.

Animals

Hyp and *Gy* mice were obtained from the Jackson Laboratory. *Gy* mice are on the B6C3H background. Normal male mice (*Y/+*) were bred with heterozygous female mice (*Gy/+*). *Hyp* mice are on the inbred C57BL/6J background. Normal male mice (*Y/+*) were bred with heterozygous female mice (*Hyp/+*). The genotype of the male littermates was determined by PCR amplification of *Phex* exons 3, 19 and 21.

Histology

To carry out histological studies, the samples were embedded in paraffin. Sections of 6 µm were prepared and stained with HE according to standard procedures.

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TITLE: Hypophosphatemic rickets: Mutation screening in the
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(1); (germany), Hyp Consortium
CORPORATE SOURCE: (1) Abt. Paediatrische Genetik, Kinderpoliklinik LMU,
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CORPORATE SOURCE: (1) Abt. Paediatr. Genet., Kinderpoliklin., LMU Muenchen,
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Angim Rishi

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Symposium 1: Molecular genetics

S1.001

The locus for a novel form of neuronal intestinal pseudoobstruction maps to Xq28

Casari Giorgio¹; Auricchio A^{1,2} (presenting); Brancolini V³; Milla P⁴; Smith VV⁴; Devoto M⁵; Ballabio A^{1,5}¹Telethon Institute of Genetics and Medicine (Tigem), San Raffaele Biomedical Science Park, Via Olgettina 58, 20132 Milan, ²Department of Pediatrics, "Federico II" University, Via Pansini 5, 80131 Naples, ³Department of Psychiatry, Columbia University, New York, New York 10032, USA, ⁴Gastroenterology Unit, Hospital for Sick Children, Great Ormond St., London England, ⁵Department of Molecular Biology, University of Siena, Italy.

Primary chronic idiopathic intestinal pseudoobstruction (CIIP) due to neuronal defects most commonly results from developmental failure of enteric neurons to correctly migrate or differentiate. This leads to clinical syndromes with varying anatomic-pathological features characterized by symptoms and signs of bowel obstruction caused by an intestinal motility disorder, in the absence of a mechanical obstacle. Most of these conditions are congenital and some are inherited. A condition characterized by intestinal pseudoobstruction with morphological abnormalities of the argyrophil neurons in the myenteric plexus, associated with short small bowel, malrotation and pyloric hypertrophy has been previously described (OMIM 243180). We have studied a family affected by this condition in which the disease appeared to segregate as an X-linked recessive trait. In order to map the CIIP locus in this family, we have performed linkage analysis in 26 family members using highly-polymorphic microsatellite markers from the X chromosome. One of these markers, DXYS154, located in the distal part of Xq28, shows no recombination with a maximum lod score of 2.32. Multipoint analysis excluded linkage with markers spanning other regions of the chromosome. Our results integrated with the current genetic and physical map of Xq28 determine the order of loci as cen-DXS15-(CIIPX)-DXS1108/DXYS154-tel. This study establishes for the first time the mapping assignment of a neuropathic form of CIIP other than Hirschsprung disease.

S1.002

*Linkage analysis to the $\alpha 2$ laminin locus on chromosome 6q2 in merosin-deficient congenital muscular dystrophy.Naom, Isam¹; D'Alessandro, M¹; Topaloglu, H²; Sewry, C¹; Helbing-Leclerc, A³; Guicheney, P³; Dubowitz, V¹; Muntoni, F¹¹Department of Paediatrics, Neuromuscular Unit, Hammersmith Hospital, London, UK, ²Department of Paediatric Neurology, Hacettepe Children's Hospital, Ankara, Turkey; ³INSERM U153, Hôpital Pitié-Salpêtrière, Paris, France.

Approximately 50% of children with the classical form of congenital muscular dystrophy (CMD) show a deficiency of one component of merosin ($\alpha 2$ laminin) in their skeletal muscle. This observation was followed by a significant advance in the understanding of CMD and establishment of the responsible gene location to a 16cM region on chromosome 6q2. We have investigated the location of the $\alpha 2$ laminin locus on chromosome 6q2 using both linkage analysis in informative families and homozygosity mapping in consanguineous families. A total of 26 merosin-deficient CMD families (9 of which were consanguineous) were typed with 13 highly polymorphic microsatellite markers spanning an area of 17cM on

chromosome 6q2. The results obtained in all the informative families (including families with partial merosin deficiency) were compatible with linkage to chromosome 6q2. Homozygosity mapping in consanguineous families and the recombinants found in informative families suggest that the actual location of the $\alpha 2$ laminin gene is more centromeric than previously thought. In particular, various recombinant events suggest that the locus is more centromeric than the D6S457 marker. Finally, linkage analysis was used in three cases of prenatal diagnosis in at-risk pregnancies. Mutation analysis of the $\alpha 2$ laminin gene is in progress in these patients.

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S1.003

*Identification of second autosomal locus predisposing to multiple deletions of mitochondrial DNA.

Kaukonen, Jyrki¹; Amati, P^{2,3}; Suomalainen A¹; Rotig A³; Piscaglia MG⁴; Salvi F⁵; Weissenbach J⁶; Fratta G⁷; Comi G⁸; Pelttonen L¹ and Zeviani M², ¹National Public Health Instit., Dept of Hum. Mol. Genet., Helsinki, Finland, ²National Neurological Instit. "Carlo Besta", Division of Biochemistry and Genetics, Milan, Italy, ³INSERM U393, Hôpital Necker-Enfants Malades, Paris, France, ⁴Division of Neurology, Public Hospital, Rimini, Italy, ⁵Hospital Bellaria, Division of Neurology, Bologna, Italy, ⁶Genethon, Evry, France, ⁷Division of Neurology, CSS-IRCCS, San Giovanni Rotondo, Foggia, Italy, ⁸Institute of Clinical Neurology, University of Milano, Italy.

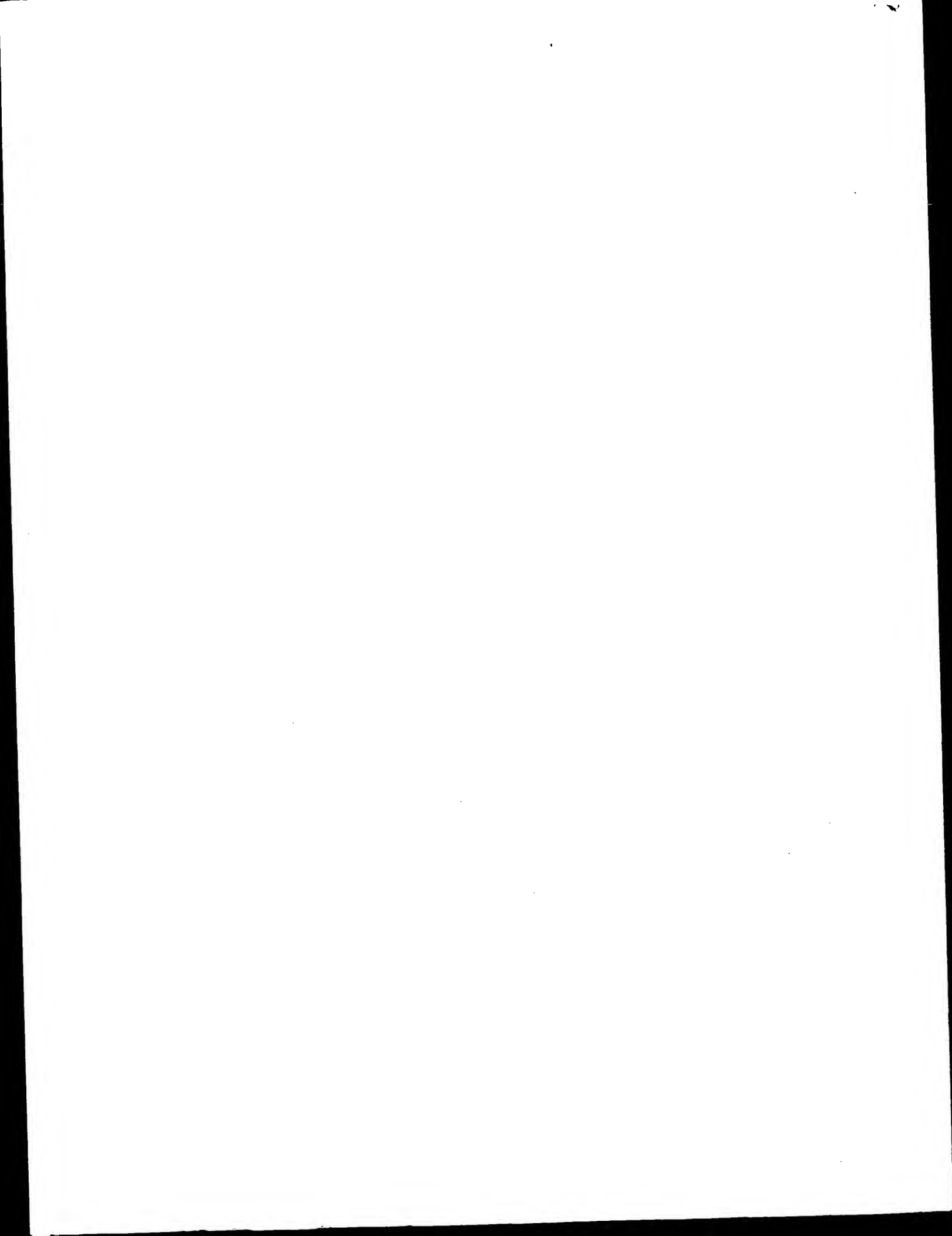
Mendelian traits leading to either qualitative or quantitative abnormalities of mtDNA are likely due to mutations in genes controlling the nuclear-mitochondrial cross-talk. A locus linked to autosomal dominant progressive external ophthalmoplegia associated with multiple mtDNA deletions (adPEO), has recently been identified on chromosome 10q in a large Finnish family (Suomalainen et al. Nature Genet. 9: 146-151, 1995). However, no linkage with the 10q locus was demonstrated in two Italian adPEO families, indicating heterogeneity. We applied random mapping approach to informative non-10q-linked Italian families to assign the second locus for adPEO. Since muscle biopsy specimens were not available from asymptomatic subjects and true affection status could thus not be verified, the linkage calculations were carried out as affected only analyses. We have now assigned the second disease locus for ADPEO within 14 cM region between markers D3S1581 and D3S1600 to chromosome 3p 14.1-21.2 in three Italian families. A maximum two-point lod score obtained was 4.62 at 0.0 with marker D3S1300. However, three adPEO families showed clear exclusion for this same region and statistical evidence for heterogeneity was obtained by the HOMOG program. These data indicate the existence of at least three adPEO loci. Assignment of the third adPEO locus is currently in progress.

S1.004

*The mouse homolog of PEX is deleted in Gy mice.

Strom, Tim M¹; Francis, F²; Econs, MJ³; Lorenz, B¹; Meindl, A¹; Rowe, PSN⁴; O'Riordan, JH⁴; Oudet, C⁵; Drezner, MK⁶; Lehrach, H⁷; Meitinger, T¹¹Abt. Pädiatrische Genetik, Kinderpoliklinik, LMU München, Goethestr. 29, 80336 München, Germany, ²MPI für Molekulare Genetik, 14195 Berlin, Germany, ³Department of Medicine, Duke University, Durham, USA, ⁴University College London, Middlesex Hospital, London, UK, ⁵IGBMC, Illkirch, France

A gene mutated in patients with X-linked hypophosphatemic rickets has been described recently by the HYP consortium. The gene named PEX was identified by a positional cloning effort in Xp22.1 and is predicted to encode a protein with homology to a family of endopeptidases including neutral endopeptidase (CALLA), endothelin converting enzyme (ECE-1) and Kell antigen. Gy mice (gyro) show hypophosphatemia, rickets and a characteristic circling behaviour. The locus is known to map to a region of the mouse X chromosome syntenic to the human PEX locus. An intragenic deletion was detected in the murine homolog using the human PEX cDNA as an hybridisation probe. A cDNA clone was obtained by screening a mouse spleen cDNA library. Sequences obtained so far show a 90% identity on protein level to the human homolog. The Gy mouse represents a model for the human disease hypophosphatemic rickets. Mutation analysis of Gy in a second hypophosphatemic mouse strain (Hyp) is currently being performed.



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331 Calcium/Phosphate and Bone Metabolism

BONE MINERAL DENSITY IN PATIENTS WITH GROWTH HORMONE DEFICIENCY AT TIME OF FINAL HEIGHT.

G. Saggese, G.I. Baroncelli, S. Barsanti, A. Rossi, C. Ceccarelli. Endocrine Unit, Dept. of Pediatrics, University of Pisa, Pisa, Italy.

Growth hormone (GH) is involved in the build-up and probably also in the maintenance of bone mass. The effect of GH treatment on bone mineral density (BMD) was examined in 11 patients (7 M, 4 F) with idiopathic GH deficiency (GHD) aged from 16.0 to 18.7 yr (17.3 ± 0.9 yr, mean \pm SD) at time they reached their final height (group A), and in 17 subjects with familial short stature aged from 16.4 to 19.8 yr (9 M, 18.5 ± 0.8 yr; 8 F 17.2 ± 0.6 yr) as controls (group B). In the past patients of group A had received discontinuous treatment with pituitary-derived human (hGH) for 43.6 ± 9.0 mo; subsequently, they had continuously received recombinant hGH for 107.3 ± 4.5 mo (prepubertal period 0.6 IU/kg/weekly, sc; pubertal period 0.9 IU/kg/weekly, sc). Total duration of GH treatment was 151.5 ± 9.7 mo. Off-treatment period was 4.7 ± 2.6 mo. In all subjects of group A and B lumbar BMDarea (L2 - L4 by DEXA, Lunar DPX-L) and lumbar BMD corrected for the estimated vertebral volume (BMDvolume) by using the formula of Kroger et al. (Bone Miner 1992; 17: 75) were measured. In patients of group A, lumbar BMDarea and lumbar BMDvolume were significantly reduced in comparison with subjects of group B (-1.2 ± 0.4 z score and -1.0 ± 0.4 z score, $p < 0.01$ and $p < 0.03$, respectively). The results show that children with GHD may have reduced BMD at time of final height. Inappropriate treatment was likely a main cause of reduced BMD. These findings suggest an important role of GH therapy in the attainment of peak bone mass in children with GHD.

332 Calcium/Phosphate and Bone Metabolism

SCHNABEL D¹, v. MÜHLENDAHL KE², MORLOT M⁴, WASSMANN A¹, GRÜTERS A¹, KRUSE K².

Depts. of Pediatrics, ¹Virchow-Klinikum, Humboldt-University Berlin, ²Medical University of Lübeck, ³Kinderhospital Osnabrück, ⁴Kinderkrankenhaus auf der Bult, Hannover, Germany. THE HEREDITARY SYNDROME OF HYPOPHOSPHATEMIC RICKETS AND HYPERCALCIURIA (HHRH): POSSIBLE DIAGNOSTIC PITFALLS AND CLINICAL FOLLOW-UP.

HHRH is a form of hypophosphatemic rickets characterized by decreased serum phosphate, reduced TmP/GFR and normocalcemia but in contrast to the x-linked hypophosphatemia (XLH) there is an appropriate increase of calcitriol ($1,25(\text{OH})_2\text{D}_3$) concentrations. This causes elevated calcium absorption, parathyroid (PTH) suppression and hypercalciuria. We report 3 patients (2f, 1m) presented at a mean age of 6 years with the following clinical features: skeletal deformities (2/3), x-ray signs of rickets (2/3) and short stature (2/3). The biochemical findings hypophosphatemia ($0.75-1.2$, normal $1.3-1.9$ mmol/l), reduced TmP/GFR ($2.0-3.1$ mg/dl), normal serum calcium and 25 -hydroxyvitamin D and low PTH-levels ($2.4-4.5$, normal $15-55$ pg/ml) resulted in the diagnosis XLH. Treatment with calcitriol and phosphate was started in 2 of 3 patients. Hypercalciuria ($254-895$, normal <188 ug/mg Cr) and signs of nephrocalcinosis questioned the diagnosis XLH. After stopping therapy the determinations of $1,25(\text{OH})_2\text{D}_3$ ($83-270$, normal $20-60$ pg/ml) and urinary calcium excretion ($292-630$ ug/mg Cr) then confirmed the diagnosis HHRH. With oral phosphate therapy alone ($50-110$ mg/kg/d) an increase of serum phosphate, a fall in plasma $1,25(\text{OH})_2\text{D}_3$ and urinary calcium excretion and healing of the rickets was achieved. After catch-up growth the patients grow now in the lower normal range. **Conclusion:** In the diagnostic work-up of hypophosphatemic rickets the determinations of urinary calcium excretion and plasma $1,25(\text{OH})_2\text{D}_3$ have to be included in order not to miss the diagnosis of HHRH.

333 Calcium/Phosphate and Bone Metabolism

HYPOPHOSPHATEMIC RICKETS: MUTATION SCREENING IN THE PEX GENE AND CLONING OF THE MOUSE HOMOLOG.

T.M. Strom¹, F. Francis², B. Lorenz¹, A. Boeddrich², M. Cagnoli³, K.L. Mohnike³, H. Lehrach², T. Meitinger¹ and the HYP Consortium. ¹Abt. Pädiatrische Genetik, Kinderpoliklinik LMU, München; ²MPI für Molekulare Genetik, Berlin; ³Zentrum für Kinderheilkunde, Magdeburg; Germany

A gene mutated in patients with X-linked hypophosphatemic rickets has been described recently by the HYP consortium¹. The gene named PEX was identified by a positional cloning effort and is predicted to encode a protein which belongs to the neutral endopeptidase family of zinc metalloproteinases including neutral endopeptidase, endothelin converting enzyme 1 and Kell antigen.

The gene spans a genomic region of about 300 kb in Xp22.1 and contains more than 22 small exons. Mutation screening by SSCP was performed for 11 exons in 26 X-linked families and 15 sporadic cases so far. 9 mutations have been detected. In addition, an intragenic breakpoint has been detected in a hypophosphatemic patient with a balanced translocation between chromosome X and 6.

In an attempt to characterise a mouse model for this disease, we have obtained mouse sequences homologous to the human PEX gene. Two mouse models for hypophosphatemia have been extensively described, the Hyp and Gy mouse. Both loci are known to map to a region of the mouse X chromosome syntenic to the human PEX locus. In Gy mice, a deletion has been detected by hybridisation of a human PEX cDNA probe. Screening of cDNA and P1 libraries in order to clone the mouse homolog of PEX is under way. Sequences obtained so far show a 90 % identity on the DNA level and a 97 % identity on the protein level to the human gene. Recovering of the full sequence should allow to screen for the mutation in the Hyp mouse and to search for the site of expression.

1. The HYP Consortium: Nature Genet 11, 130-136 (1995).

334 Calcium/Phosphate and Bone Metabolism

RELATIVE OSTEOPENIA FOLLOWING TREATMENT FOR CHILDHOOD ACUTE LYMPHOBLASTIC LEUKAEMIA.

J.T. Warner, W.D. Evans, F.D.J. Dunstan, D.K.H. Webb, J.W. Gregory. Departments of Child Health, Medical Physics and Medical Statistics, University of Wales College of Medicine and Llandough Hospital NHS Trust, Cardiff, U.K.

Introduction: Osteopenia as a result of growth hormone deficiency (GHD) following cranial radiotherapy (CRT) is now recognised. Low dose CRT, 18-24 Gy is not often associated with frank GHD yet subtle changes in pulsatility have been described. The aim of this study was to compare bone mineralisation in long term survivors of childhood acute lymphoblastic leukaemia (ALL), treated with chemotherapy and low dose CRT, with both survivors of other malignancies (OM), who had received chemotherapy alone, and with healthy sibling controls.

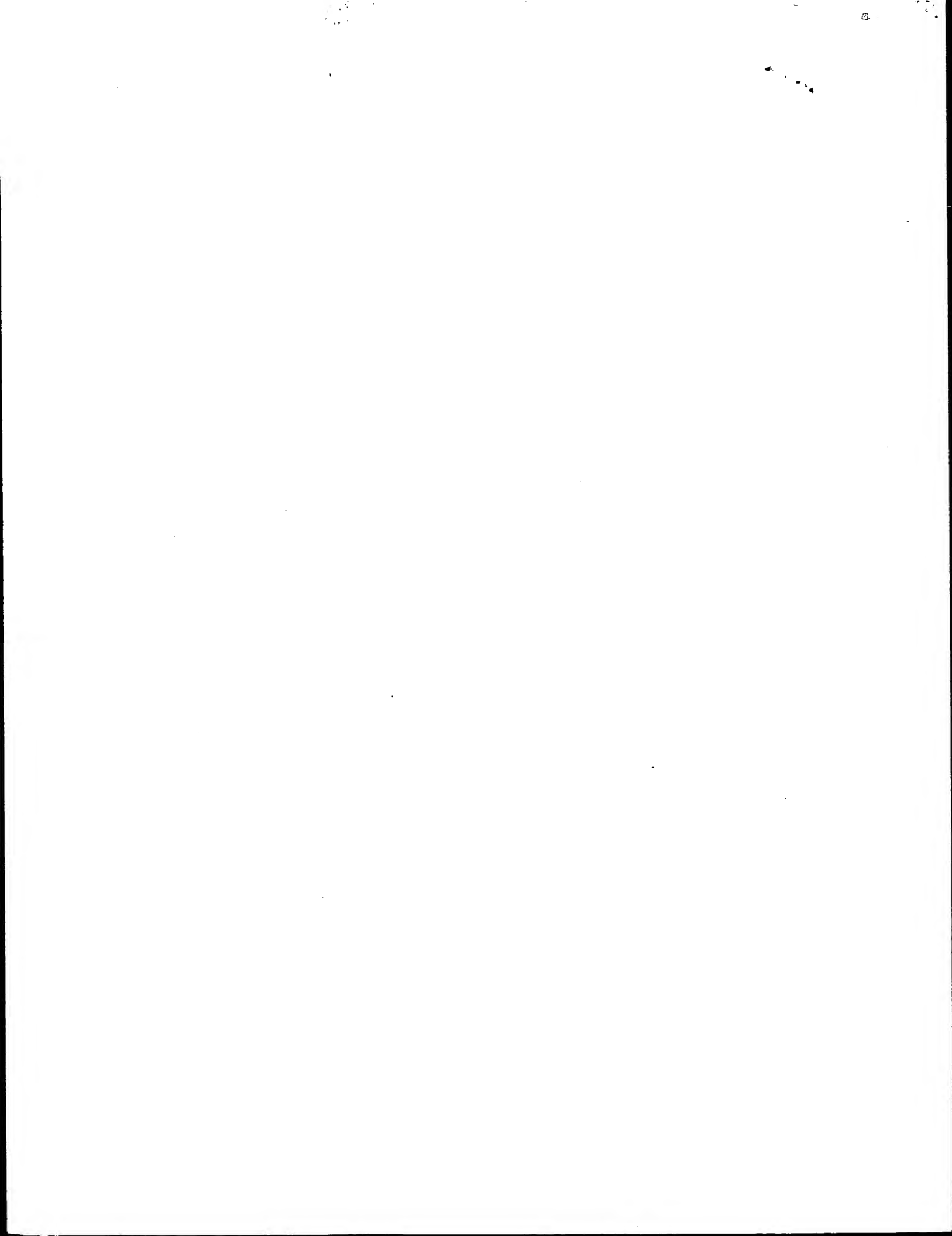
Patients and methods: Bone mineral content (BMC, grams) and bone area (BA, cm²) of the whole body (WB), lumbar spine (LS) and left hip were measured by dual energy X-ray absorptiometry in 34 (13 male) survivors of ALL (7.2-18.2 yrs), 20 (10 male) OM survivors (7.3-18.4 yrs), and 30 (16 male) healthy siblings (7.6-17.3 yrs). Bone mineral density (BMD) at each site was calculated from BMC/BA where p is the power coefficient derived from regressing BMC on BA in controls after conversion to natural logs ($r=0.99, 0.95$ and 0.95 for WB, LS and hip respectively). A predicted BMD was derived from controls based on height and pubertal stage for LS and hip, with addition of age and gender for the WB. The measured BMD was expressed as a percentage of predicted (%BMD).

Results: Survivors of ALL and OM had completed treatment $6.4[2.6-12.8]$ and $6.7[1.5-11.8]$ mean [range] years respectively. Mean %BMD [SD] are given in the table. 21% of survivors of ALL had a %BMD of less than -1.0 SD for all sites compared with 5% of the OM group and 3% of controls.

	WB	LS	Hip
Sibs	100.0 [4.0]	100.0 [9.9]	100.0 [9.3]
ALL	96.8 [4.8]†	92.2 [7.9]†	88.8 [10.7]†
OM	97.0 [3.5]†	96.8 [10.6]†	95.4 [11.2]†

(† $p < 0.01$ cf. sibs, ‡ $p < 0.05$ cf. OM, § $p < 0.001$ cf. OM, ¶ NS cf. sibs)

Conclusion: Children treated for ALL have a significantly reduced BMD at all sites compared to healthy siblings. Reduction in BMD in OM was not significant except for WB. These findings suggest that CRT (perhaps as a consequence of subtle abnormalities of GH release) and to a lesser extent chemotherapy (possibly as a result of corticosteroid therapy) predispose to long term osteopenia.



Rishi, Anjum

From: Rishi, Anjum
Sent: Tuesday, January 15, 2002 9:46 AM
To: STIC-ILL
Subject: ILL Order, CM 1 MailRoom 12th Floor, Ph# 308-4422, Case # 09/806/110

L5 ANSWER 10 OF 29 EMBASE COPYRIGHT 2002 ELSEVIER SCI. B.V.DUPLICATE 2

ACCESSION NUMBER: 1998194311 EMBASE

TITLE: Cloning of human PEX cDNA: Expression,
subcellular localization, and endopeptidase activity.

AUTHOR: Lipman M.L.; Panda D.; Bennett H.P.J.; Henderson J.E.;
Shane E.; Shen Y.; Goltzman D.; Karaplis A.C.

CORPORATE SOURCE: A.C. Karaplis, Div. of Endocrinology, Sir M.B. Davis-Jewish
Gen. Hospital, McGill University, 3755 Cote Ste-Catherine
Rd., Montreal, Que. H3T 1E2, Canada.
akarapli@ldi.jgh.mcgill.ca

SOURCE: Journal of Biological Chemistry, (29 May 1998) 273/22
(13729-13737).

Refs: 35

ISSN: 0021-9258 CODEN: JBCHA3

COUNTRY: United States

DOCUMENT TYPE: Journal; Article

FILE SEGMENT: 029 Clinical Biochemistry

LANGUAGE: English

SUMMARY LANGUAGE: EnglishL5 ANSWER 11 OF 29 EMBASE COPYRIGHT 2002 ELSEVIER SCI.
B.V.DUPLICATE 3

ACCESSION NUMBER: 1998385394 EMBASE

TITLE: Genetic screening for X-linked hypophosphatemic mice and
ontogenic characterization of the defect in the renal
sodium-phosphate transporter.

AUTHOR: Muller Y.L.; Collins J.F.; Ghishan F.K.

CORPORATE SOURCE: Dr. F.K. Ghishan, Department of Pediatrics, Steele Memorial
Children's Res. Ctr., Univ. of Arizona Hlth. Sci. Center,
1501 N. Campbell Avenue, Tucson, AZ 85724, United States

SOURCE: Pediatric Research, (1998) 44/5 (633-638).

Refs: 18

ISSN: 0031-3998 CODEN: PEREBL

COUNTRY: United States

DOCUMENT TYPE: Journal; Article

FILE SEGMENT: 007 Pediatrics and Pediatric Surgery

022 Human Genetics

029 Clinical Biochemistry

LANGUAGE: English

SUMMARY LANGUAGE: EnglishL5 ANSWER 12 OF 29 EMBASE COPYRIGHT 2002 ELSEVIER SCI.
B.V.DUPLICATE 4

ACCESSION NUMBER: 1998086348 EMBASE

TITLE: Spermine deficiency in Gy mice caused by deletion of the
spermine synthase gene.

AUTHOR: Lorenz B.; Francis F.; Gempel K.; Bsddrich A.; Josten M.;
Schmahl W.; Schmidt J.; Lehrach H.; Meitinger T.; Strom
T.M.

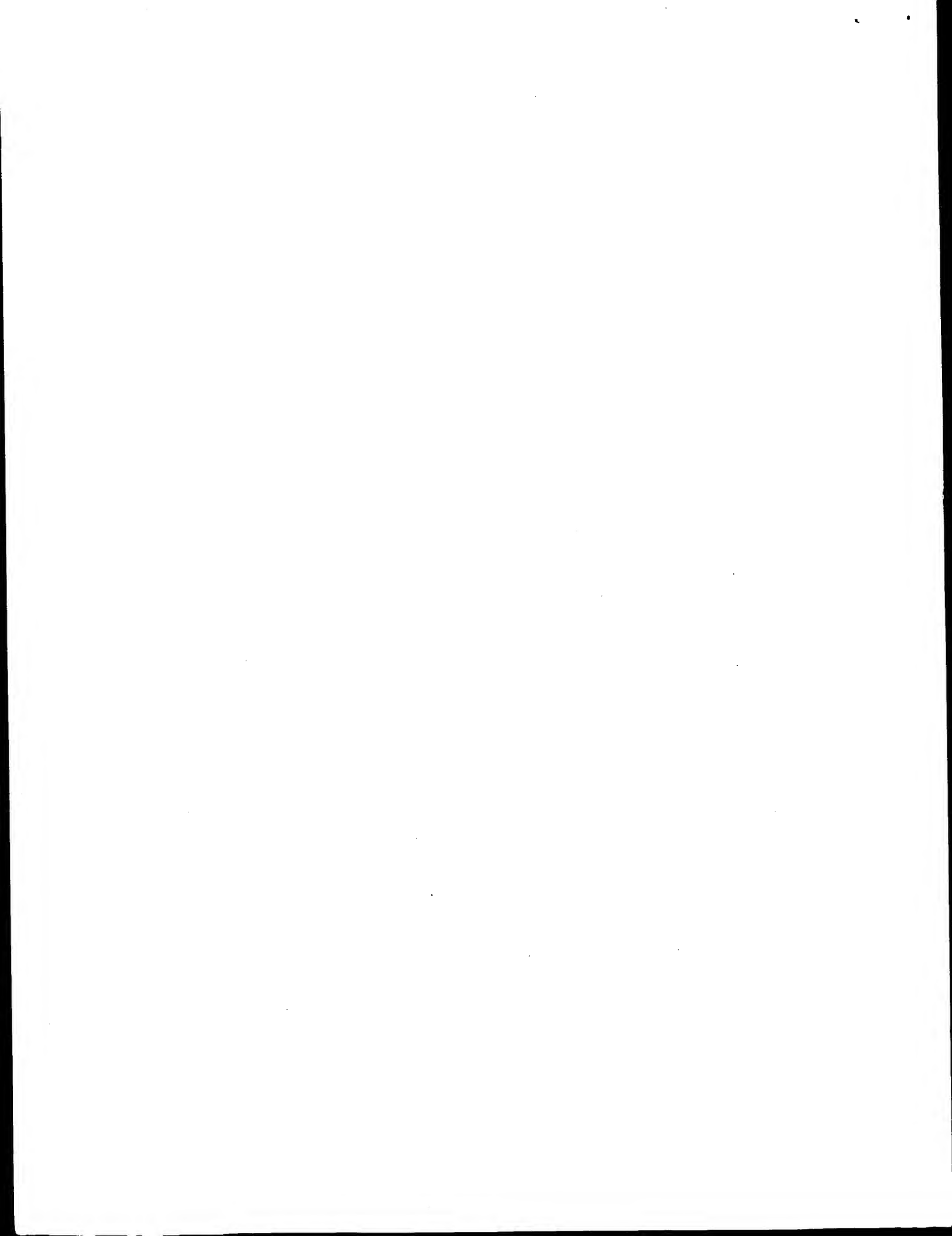
CORPORATE SOURCE: T.M. Strom, Abteilung Medizinische Genetik,
Kinderpoliklinik, Ludwig-Maximilians-Universitat,
Goethestr. 29, 80336 Munchen, Germany.
timstrom@pedgen.med.uni-muenchen.de

SOURCE: Human Molecular Genetics, (1998) 7/3 (541-547).

Refs: 34

ISSN: 0964-6906 CODEN: HMGEE5

COUNTRY: United Kingdom
 DOCUMENT TYPE: Journal; Article
 FILE SEGMENT: 022 Human Genetics
 LANGUAGE: English
 SUMMARY LANGUAGE: English L5 ANSWER 13 OF 29 BIOSIS COPYRIGHT 2002 BIOSIS
 ACCESSION NUMBER: 1998:227575 BIOSIS
 DOCUMENT NUMBER: PREV199800227575
 TITLE: Pex mRNA is localized in developing mouse osteoblasts and odontoblasts.
 AUTHOR(S): Ruchon, Andrea Frota; Marcinkiewicz, Mieczyslaw; Siefried, Geraldine; Tenenhouse, Harriet S.; Desgroseiller, Luc; Crine, Philippe; Boileau, Guy (1)
 CORPORATE SOURCE: (1) Dep. Biochimie, Univ. Montreal, CP 6128, Succ. Centre-Ville, Montreal Qc H3C 3J7 Canada
 SOURCE: Journal of Histochemistry and Cytochemistry, (April, 1998) Vol. 46, No. 4, pp. 459-468.
 ISSN: 0022-1554.
 DOCUMENT TYPE: Article
 LANGUAGE: English
 L5 ANSWER 15 OF 29 BIOSIS COPYRIGHT 2002 BIOSIS
 ACCESSION NUMBER: 1998:228230 BIOSIS
 DOCUMENT NUMBER: PREV199800228230
 TITLE: Molecular analysis of peroxisomal disorders.
 AUTHOR(S): Shimozawa, Nobuyuki (1)
 CORPORATE SOURCE: (1) Dep. Pediatr., Gifu Univ. Sch. Med., Gifu Japan
 SOURCE: No To Hattatsu, (March, 1998) Vol. 30, No. 2, pp. 129-133.
 ISSN: 0029-0831.
 DOCUMENT TYPE: Article
 LANGUAGE: Japanese
 SUMMARY LANGUAGE: Japanese; English ANSWER 17 OF 29 EMBASE COPYRIGHT 2002 ELSEVIER SCI. B.V. DUPLICATE 6
 ACCESSION NUMBER: 1998148625 EMBASE
 TITLE: Cellular/molecular control of renal Na/P(i)-cotransport.
 AUTHOR: Murer H.; Forster I.; Hilfiker H.; Pfister M.; Kaissling B.; Lotscher M.; Biber J.
 CORPORATE SOURCE: Dr. H. Murer, Institute of Physiology, Switzerland Physiologisches Institut, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland. murer@physiol.unizh.ch
 SOURCE: Kidney International, Supplement, (1998) 53/65 (S2-S10).
 Refs: 84
 ISSN: 0098-6577 CODEN: KISUDF
 COUNTRY: United States
 DOCUMENT TYPE: Journal; Conference Article
 FILE SEGMENT: 002 Physiology
 028 Urology and Nephrology
 LANGUAGE: English
 SUMMARY LANGUAGE: English
 L5 ANSWER 19 OF 29 EMBASE COPYRIGHT 2002 ELSEVIER SCI. B.V.
 ACCESSION NUMBER: 97099288 EMBASE
 DOCUMENT NUMBER: 1997099288
 TITLE: Pex/PEX tissue distribution and evidence for a deletion in the 3' region of the Pex gene in X-linked hypophosphatemic mice.
 AUTHOR: Beck L.; Soumounou Y.; Martel J.; Krishnamurthy G.; Gauthier C.; Goodyer C.G.; Tenenhouse H.S.
 CORPORATE SOURCE: H.S. Tenenhouse, Montreal Children's Hospital, 2300 Tupper Street, Montreal, Que. H3H 1P3, United States.
 mdht@musica.mcgill.ca
 SOURCE: Journal of Clinical Investigation, (1997) 99/6 (1200-1209).
 Refs: 40
 ISSN: 0021-9738 CODEN: JCINAO
 COUNTRY: United States



DOCUMENT TYPE: Journal; Article
FILE SEGMENT: 005 General Pathology and Pathological Anatomy
033 Orthopedic Surgery
LANGUAGE: English
SUMMARY LANGUAGE: EnglishL5 ANSWER 20 OF 29 EMBASE COPYRIGHT 2002 ELSEVIER SCI. B.V.DUPLICATE 7
ACCESSION NUMBER: 1998032157 EMBASE
TITLE: Oncogenic osteomalacia: Is there a new phosphate regulating hormone?
AUTHOR: Nelson A.E.; Robinson B.G.; Mason R.S.
CORPORATE SOURCE: Dr. A.E. Nelson, Molecular Genetics Department, Kolling Inst. of Medical Research, Royal North Shore Hospital, St Leonards, NSW 2065, Australia. annen@med.usyd.edu.au
SOURCE: Clinical Endocrinology, (1997) 47/6 (635-642).
Refs: 68
ISSN: 0300-0664 CODEN: CLENAO
COUNTRY: United Kingdom
DOCUMENT TYPE: Journal; General Review
FILE SEGMENT: 003 Endocrinology
LANGUAGE: English
SUMMARY LANGUAGE: EnglishNSWER 21 OF 29 EMBASE COPYRIGHT 2002 ELSEVIER SCI. B.V.DUPLICATE 8
ACCESSION NUMBER: 97346024 EMBASE
DOCUMENT NUMBER: 1997346024
TITLE: Positional cloning of the PEX gene: New insights into the pathophysiology of X-linked hypophosphatemic rickets.
AUTHOR: Econs M.J.; Francis F.
CORPORATE SOURCE: M.J. Econs, Dept. of Medicine, Indiana University Medical Center, 975 W. Walnut St., Indianapolis, IN 46202, United States
SOURCE: American Journal of Physiology - Renal Physiology, (1997) 273/4 42-4 (F489-F498).
Refs: 90
ISSN: 0363-6127 CODEN: AJPPFK
COUNTRY: United States
DOCUMENT TYPE: Journal; General Review
FILE SEGMENT: 005 General Pathology and Pathological Anatomy
033 Orthopedic Surgery
LANGUAGE: English
SUMMARY LANGUAGE: EnglishL5 ANSWER 22 OF 29 EMBASE COPYRIGHT 2002 ELSEVIER SCI. B.V.
ACCESSION NUMBER: 97132353 EMBASE
DOCUMENT NUMBER: 1997132353
TITLE: New perspectives on the biology and treatment of X-linked hypophosphatemic rickets.
AUTHOR: Carpenter T.O.
CORPORATE SOURCE: Dr. T.O. Carpenter, Department of Pediatrics, Yale University School of Medicine, New Haven, CT 06520-8064, United States
SOURCE: Pediatric Clinics of North America, (1997) 44/2 (443-466).
Refs: 109
ISSN: 0031-3955 CODEN: PCNAA8
COUNTRY: United States
DOCUMENT TYPE: Journal; General Review
FILE SEGMENT: 007 Pediatrics and Pediatric Surgery
022 Human Genetics
LANGUAGE: EnglishL5 ANSWER 24 OF 29 EMBASE COPYRIGHT 2002 ELSEVIER SCI. B.V.DUPLICATE 9
ACCESSION NUMBER: 97054291 EMBASE
DOCUMENT NUMBER: 1997054291
TITLE: Per gene deletions in Gy and Hyp mice provide mouse models for X-linked hypophosphatemia.
AUTHOR: Strom T.M.; Francis F.; Lorenz B.; Boddich A.; Econs M.J.; Lehrach H.; Meitinger T.

CORPORATE SOURCE: T.M. Strom, Abteilung Medizinische Genetik,
Kinderpoliklinik, Ludwig-Maximilians-Universitat, Goethestr
29, 80336 Munchen, Germany

SOURCE: Human Molecular Genetics, (1997) 6/2 (165-171).
Refs: 34
ISSN: 0964-6906 CODEN: HMGEE5

COUNTRY: United Kingdom

DOCUMENT TYPE: Journal; Article

FILE SEGMENT: 022 Human Genetics
029 Clinical Biochemistry

LANGUAGE: English

SUMMARY LANGUAGE: EnglishL5 ANSWER 25 OF 29 BIOSIS COPYRIGHT 2002 BIOSIS

ACCESSION NUMBER: 1996:500493 BIOSIS

DOCUMENT NUMBER: PREV199699222849

TITLE: Recently clones genes involved in calcium and phosphate
homeostasis.

AUTHOR(S): Strom, Tim M. (1); Francis, Fiona

CORPORATE SOURCE: (1) Abt. Paediatriche Genetik, Kinderpoliklinik,
Ludwig-Maximilians-Univ., Goethestr. 29, 80336 Muenchen
Germany

SOURCE: Schoenau, E. [Editor]. International Congress Series,
(1996) No. 1105, pp. 53-58. International Congress Series;
Paediatric osteology: New developments in diagnostics and
therapy.
Publisher: Elsevier Science Publishers B.V. PO Box 211,
Sara Burgerhartstraat 25, 1000 AE Amsterdam, Netherlands.
Meeting Info.: First International Workshop on Paediatric
Osteology Cologne, Germany October 5-7, 1995
ISSN: 0531-5131. ISBN: 0-444-82277-1.

DOCUMENT TYPE: Book; Conference

LANGUAGE: EnglishL5 ANSWER 26 OF 29 BIOSIS COPYRIGHT 2002 BIOSIS

ACCESSION NUMBER: 1996:497912 BIOSIS

DOCUMENT NUMBER: PREV199699220268

TITLE: Hypophosphatemic rickets: Mutation screening in the
PEX gene and cloning of the mouse homolog.

AUTHOR(S): Strom, T. M. (1); Francis, F.; Lorenz, B. (1); Boeddrich,
A.; Cagnoli, M.; Mohnike, K. L.; Lehrach, H.; Meitinger, T.
(1); (germany), Hyp Consortium

CORPORATE SOURCE: (1) Abt. Paediatriche Genetik, Kinderpoliklinik LMU,
Muenchen Germany

SOURCE: Hormone Research (Basel), (1996) Vol. 46, No. SUPPL. 2, pp.
84.
Meeting Info.: 35th Annual Meeting of the European Society
for Paediatric Endocrinology Montpellier, France September
15-18, 1996
ISSN: 0301-0163.

DOCUMENT TYPE: Conference

LANGUAGE: English5 ANSWER 27 OF 29 BIOSIS COPYRIGHT 2002 BIOSIS

ACCESSION NUMBER: 1996:355236 BIOSIS

DOCUMENT NUMBER: PREV199699077592

TITLE: The mouse homolog of PEX is deleted in Gy mice.

AUTHOR(S): Strom, Tim M. (1); Francis, F.; Econs, M. J.; Lorenz, B.
(1); Meindl, A. (1); Rowe, P. S. N.; O'Riordan, J. L. H.;
Oudet, C.; Drezner, M. K.; Lehrach, H.; Meitinger, T. (1)

CORPORATE SOURCE: (1) Abt. Paediatr. Genet., Kinderpoliklin., LMU Muenchen,
Goethestr. 29, 80336 Muenchen Germany

SOURCE: European Journal of Human Genetics, (1996) Vol. 4, No.
SUPPL. 1, pp. 1.
Meeting Info.: 28th Annual Meeting of the European Society
of Human Genetics London, England, UK April 11-13, 1996
ISSN: 1018-4813.

DOCUMENT TYPE: Conference

LANGUAGE: EnglishL5 ANSWER 28 OF 29 EMBASE COPYRIGHT 2002 ELSEVIER SCI. B.V.DUPLICATE 10

ACCESSION NUMBER: 94135864 EMBASE

DOCUMENT NUMBER: 1994135864

TITLE: Production of human parathyroid hormone
by recombinant Escherichia coli TG1 on synthetic medium.

AUTHOR: Harder M.P.F.; Sanders E.A.; Wingender E.; Deckwer W.-D.

CORPORATE SOURCE: GBF, Gesellsch. fur Biotechn. Forsch. mbH, Mascheroder Weg
1,D-38124 Braunschweig, Germany

SOURCE: Journal of Biotechnology, (1994) 32/2 (157-164).

ISSN: 0168-1656 CODEN: JBITD4

COUNTRY: Netherlands

DOCUMENT TYPE: Journal; Article

FILE SEGMENT: 004 Microbiology

029 Clinical Biochemistry

037 Drug Literature Index

LANGUAGE: English

SUMMARY LANGUAGE: EnglishL5 ANSWER 28 OF 29 EMBASE COPYRIGHT 2002 ELSEVIER SCI.
B.V.DUPLICATE 10

ACCESSION NUMBER: 94135864 EMBASE

DOCUMENT NUMBER: 1994135864

TITLE: Production of human parathyroid hormone
by recombinant Escherichia coli TG1 on synthetic medium.

AUTHOR: Harder M.P.F.; Sanders E.A.; Wingender E.; Deckwer W.-D.

CORPORATE SOURCE: GBF, Gesellsch. fur Biotechn. Forsch. mbH, Mascheroder Weg
1,D-38124 Braunschweig, Germany

SOURCE: Journal of Biotechnology, (1994) 32/2 (157-164).

ISSN: 0168-1656 CODEN: JBITD4

COUNTRY: Netherlands

DOCUMENT TYPE: Journal; Article

FILE SEGMENT: 004 Microbiology

029 Clinical Biochemistry

037 Drug Literature Index

LANGUAGE: English

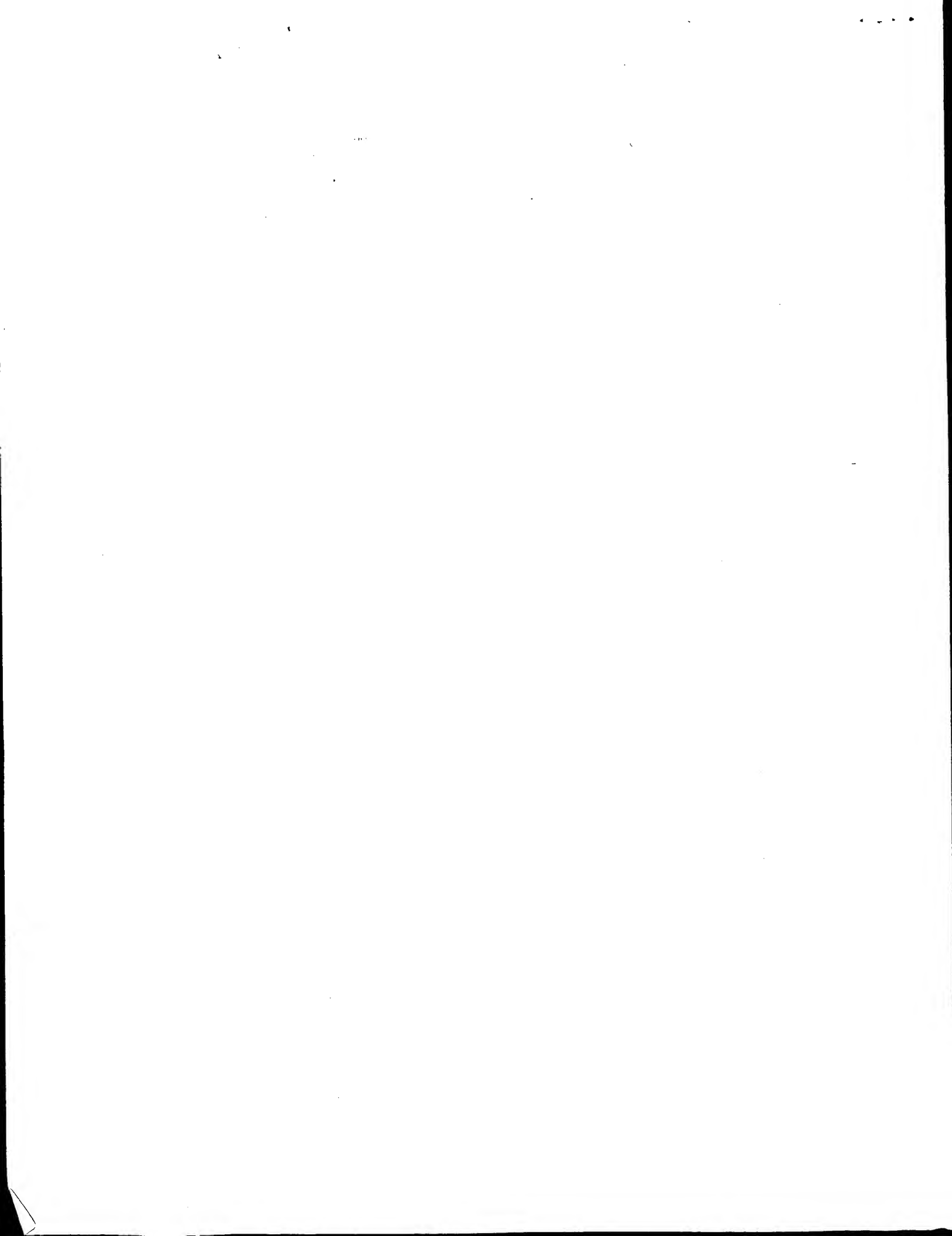
SUMMARY LANGUAGE: English

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osteoblasts and odontoblasts.
AUTHOR(S): Ruchon, Andrea Frota; Marcinkiewicz, Mieczyslaw; Siefried, Geraldine; Tenenhouse, Harriet S.; Desgroseiller, Luc; Crine, Philippe; Boileau, Guy (1)
CORPORATE SOURCE: (1) Dep. Biochimie, Univ. Montreal, CP 6128, Succ. Centre-Ville, Montreal Qc H3C 3J7 Canada
SOURCE: Journal of Histochemistry and Cytochemistry, (April, 1998) Vol. 46, No. 4, pp. 459-468.
ISSN: 0022-1554.

DOCUMENT TYPE: Article

LANGUAGE: English

L5 ANSWER 15 OF 29 BIOSIS COPYRIGHT 2002 BIOSIS

ACCESSION NUMBER: 1998:228230 BIOSIS

DOCUMENT NUMBER: PREV199800228230

TITLE: Molecular analysis of peroxisomal disorders.

AUTHOR(S): Shimozawa, Nobuyuki (1)

CORPORATE SOURCE: (1) Dep. Pediatr., Gifu Univ. Sch. Med., Gifu Japan

SOURCE: No To Hattatsu, (March, 1998) Vol. 30, No. 2, pp. 129-133.

ISSN: 0029-0831.

DOCUMENT TYPE: Article

LANGUAGE: Japanese

SUMMARY LANGUAGE: Japanese; English ANSWER 17 OF 29 EMBASE COPYRIGHT 2002 ELSEVIER SCI. B.V.DUPLICATE 6

ACCESSION NUMBER: 1998148625 EMBASE

TITLE: Cellular/molecular control of renal Na/P(i)-cotransport.

AUTHOR: Murer H.; Forster I.; Hilfiker H.; Pfister M.; Kaissling

B.; Lotscher M.; Biber J.

CORPORATE SOURCE: Dr. H. Murer, Institute of Physiology, Switzerland

Physiologisches Institut, Winterthurerstrasse 190, CH-8057

Zurich, Switzerland. murer@physiol.unizh.ch

SOURCE: Kidney International, Supplement, (1998) 53/65 (S2-S10).

Refs: 84

ISSN: 0098-6577 CODEN: KISUDF

COUNTRY: United States

DOCUMENT TYPE: Journal; Conference Article

FILE SEGMENT: 002 Physiology

028 Urology and Nephrology

LANGUAGE: English

SUMMARY LANGUAGE: English

L5 ANSWER 19 OF 29 EMBASE COPYRIGHT 2002 ELSEVIER SCI. B.V.

ACCESSION NUMBER: 97099288 EMBASE

DOCUMENT NUMBER: 1997099288

TITLE: Pex/PEX tissue distribution and evidence for a deletion in the 3' region of the Pex gene in X-linked hypophosphatemic mice.

AUTHOR: Beck L.; Soumounou Y.; Martel J.; Krishnamurthy G.;

Gauthier C.; Goodyer C.G.; Tenenhouse H.S.

CORPORATE SOURCE: H.S. Tenenhouse, Montreal Children's Hospital, 2300 Tupper Street, Montreal, Que. H3H 1P3, United States.

mdht@musica.mcgill.ca

SOURCE: Journal of Clinical Investigation, (1997) 99/6 (1200-1209).

Refs: 40

ISSN: 0021-9738 CODEN: JCINAO

COUNTRY: United States

DOCUMENT TYPE: Journal; Article

FILE SEGMENT: 005 General Pathology and Pathological Anatomy

033 Orthopedic Surgery

LANGUAGE: English

SUMMARY LANGUAGE: EnglishL5 ANSWER 20 OF 29 EMBASE COPYRIGHT 2002 ELSEVIER SCI. B.V.DUPLICATE 7

ACCESSION NUMBER: 1998032157 EMBASE

TITLE: Oncogenic osteomalacia: Is there a new phosphate regulating hormone?

AUTHOR: Nelson A.E.; Robinson B.G.; Mason R.S.

CORPORATE SOURCE: Dr. A.E. Nelson, Molecular Genetics Department, Kolling Inst. of Medical Research, Royal North Shore Hospital, St

Leonards, NSW 2065, Australia. annen@med.usyd.edu.au

SOURCE: Clinical Endocrinology, (1997) 47/6 (635-642).

Refs: 68

ISSN: 0300-0664 CODEN: CLENAO

COUNTRY: United Kingdom

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09806110

1/15 1072

R.B.J.S.
NPL.
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